Overview of Receptors from Combinatorial Nucleic Acid and Protein Libraries

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ABSTRACT

This unit provides a brief description of the different approaches that can be used to identify functional peptides, proteins, and nucleic acids from combinatorial libraries. *Curr. Protoc. Mol. Biol.* 80:24.1.1-24.1.3. © 2007 by John Wiley & Sons, Inc.

Keywords: combinatorial library • protein • nucleic acid • aptamer • selection • SELX • phage display • directed evolution

Biopolymer receptors are used in a wide variety of molecular biology techniques, from ELISAs to immunoprecipitations to proteomic arrays. Receptors that are of particular interest or utilty for these applications can be generated by either selection or by screening combinatorial libraries.

Combinatorial library methods can be roughly classified according to the type of molecules being examined and to the deconvolution methods being used. In particular, one large division is between biopolymer and chemical libraries, while a second division is between selections and screens for function. Selections rely on the amplification of templates encoding functional receptors, while screens rely on the identification and subsequent resynthesis of functional receptors. As a rough generalization, biopolymer libraries are frequently selected for function (although they can also be screened), while chemical libraries are frequently screened for function (although with increasingly novel methods they can be selected).

Biopolymer libraries include, but are not limited to, proteins and nucleic acids. Protein libraries can be of many different forms, from the partial randomization of large proteins to the segmental randomization of pieces of proteins, to the complete randomization of peptides. Since proteins do not encode replicable sequence information, proteins and their attendant phenotypes must somehow be coupled to genetic sequence information, i.e., RNA or DNA. There are several different methods by which this can be accomplished, but in all instances the key is the physical link between phenotype and genotype. In one of the first and most robust instantiations of protein selection methods, peptide libraries were adjoined to phage proteins such as pIII and pVIII, and thereby expressed ("displayed") on the surface of bacteriophage. Such phage libraries tend to have on the order of a billion different variants. Selection for binding led to the isolation of peptides that carried with them the genes that encoded them. Re-infection of cells led to the amplification of phage with these desirable phenotypes. Multiple cycles of selection and amplification generally led to the purification of phage peptides that could bind to a given target. Oftentimes, however, binding required the multivalent presentation of the peptides (i.e., researchers got exactly what they selected for: the peptides were presented in several copies, and the best binding phage used these several copies to interact with a target). Since these first demonstrations, phage display methods have been devised for the selection of antibodies, enzyme substrates, and enzymes themselves. Phage display selection is the subject of protocols in other *Current Protocols* volumes (e.g., Bradbury, 1999; Galanis et al., 1999; Benhar and Reiter, 2002; Bradbury et al., 2002; Enshell-Seijffers and Gershoni, 2002; Kay and Castagnoli, 2003) and has been reviewed many times in the literature (Kehoe and Kay, 2005). In addition, other viruses and entire cells have been used as vehicles for the display of protein libraries (see, for example, Farinas, 2006).

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In addition to selecting peptides or proteins displayed on the outside of a cell or phage, peptides or proteins can be selected within cells. There is a long history of carrying out directed evolution experiments with whole cells, based in large measure on cellular phenotypes and natural or artificially elevated mutation rates. One classic example is the selection of evolved beta galactosidase (ebg; Hall, 2003). However, the ability to chemically manipulate DNA and thereby create DNA libraries drastically increased the ability to select nucleic acids with novel phenotypes. In recent years, so-called peptide aptamers (a term originally applied to nucleic acids, see below) have been selected based on the ability of individual library members to inhibit protein functions, such as enzymatic activity or dimerization, and thereby modulate key features of cell physiology, such as signal transduction pathways (numerous examples can be found in Hoppe-Seyler et al., 2004; Baines and Colas, 2006). While almost any phenotype can be screened or selected, it is frequently useful to couple peptide aptamer function to the production of a contrived genetic marker, such as an antibiotic or fluorescent protein. While the great advantage of peptide aptamers is their immediate tie to a relevant cellular phenotype, the library sizes that can be examined are limited by transformation efficiencies and cell-based selection methods to generally $\leq 10^7$. The production and utility of peptide aptamers is examined in greater detailin *UNIT 24.4*.

In all of these instances, translation inside of a cell has been used to generate a protein library. There are also methods where translation outside of a cell can be used to generate libraries in which phenotype is connected with genotype. There are several popular variants of in vitro display technologies: ribosome or mRNA display (Lipovsek and Pluckthun, 2004), and in vitro compartmentalization (Rothe et al., 2006). In ribosome display, the elimination of a stop codon or release factor leads to mRNAs being noncovalently linked to peptides or proteins extruded through the exit pore of the ribosome. The entire complex can be selected for binding or other functions. In mRNA display, the antibiotic puromycin, which normally covalently adds to growing peptide chains, is linked to a nucleic acid, causing the nucleic acid to be covalently added to a growing peptide or protein chain. Again, an mRNA is connected to its translated protein counterpart, except in this instance the connection is via a covalent linkage rather than a noncovalent one. An excellent description of mRNA display can be found in *UNIT 24.5*. Finally, in vitro compartmentalization methods utilize in vitro transcription and translation mixes in water-in-oil emulsions to generate literally billions of separate 'cell-like' compartments where individual proteins in a library can be made. In this instance, the connection between genotype and phenotype is initially enforced by the compartment itself. Clever schemes to further enforce the linkage have also been devised, e.g., a gene that is covalently coupled to a bead produces an enzyme that fluorescently labels the bead, which is in turn captured via FACS. Amplification of the gene allows further cycles of selection for those enzymes that are most active and those beads that are most fluorescent. While these methods are quite different from one another, in the libraries that can be sieved are in general larger ($\geq 10^{10}$) than is the case with phage display (Griffiths and Tawfik, 2006).

Functional nucleic acids can also be selected from random sequence libraries. In these instances, the coupling between genotype and phenotype is natural, since functional nucleic acids overcome the 'chicken and egg' problem: the genotype is the phenotype, and vice versa. Individual, single-stranded nucleic acids (DNA or RNA) can be generated by either chemical or enzymatic methods. The current volume contains a detailed description of how to prepare a nucleic acid pool (*UNIT 24.1*). Each single-stranded nucleic acid will fold into a unique three-dimensional conformation. These conformations can be sieved for either binding or catalytic activity (ribozymes). Nucleic acid variants that survive a round of selection can be amplified by a combination of reverse transcription, PCR, and in vitro transcription. One of the more common and useful types of in vitro selection experiments is the identification of anti-protein aptamers via filter-binding selection, a procedure that is described in *UNIT 24.3*. The disadvantage of using nucleic acid libraries is that the chemistry is not nearly as robust as for proteins: the 5 canonical nucleobases have much less chemical functionality than the 20 amino acids. This disadvantage is being overcome by the inclusion of modified nucleotides during enzymatic replication or transcription. The advantage of using nucleic acid libraries is that they can be much larger than protein libraries (on the order of 10^{15} variants) and can be manipulated entirely in vitro. Nucleic acid selections are increasingly yielding aptamers with biomedical

relevance, as reviewed in Nimjee et al. (2005) and Yan et al. (2005). While most nucleic acid selections are carried out in vitro, it has also proven possible to directly select for function in vivo, as with peptide aptamers (Cassidy and Mahler, 2003).

It is anticipated that the line between chemistry and biology will become increasingly blurred. Already, it has proven possible to generate chemical libraries with nucleic acid or peptide tags, allowing the details regarding the composition and synthesis of a given compound to be encoded in a biopolymer (Brenner and Lerner, 1992). While such methods can simplify the identification of active pharmacophores, they do not yield replicable chemical compounds per se, as delimited chemical libraries must still be resynthesized based on the functional information gained from a given round of screening or selection. However, more recently small chemical libraries have been synthesized based on the alignment of reactive chemical compounds on DNA templates (Gartner et al., 2004; Scheuermann et al., 2006). By coupling DNA tagging and DNA templating methodologies, it has even proven possible to directly evolve the structures of chemical compounds (Halpin and Harbury, 2004).

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Design, Synthesis, and Amplification of DNA Pools for In Vitro Selection

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ABSTRACT

Preparation of a random-sequence DNA pool is presented. The degree of randomization and the length of the random sequence are discussed, as is synthesis of the pool using a DNA synthesizer or via commercial synthesis companies. Purification of a single-stranded pool and conversion to a double-stranded pool are presented as step-by-step protocols. Support protocols describe determination of the complexity and skewing of the pool, and optimization of amplification conditions. *Curr. Protoc. Mol. Biol.* 88:24.2.1-24.2.27. © 2009 by John Wiley & Sons, Inc.

Keywords: In vitro selection • DNA pool synthesis • phosphoramidite DNA synthesis • randomization

INTRODUCTION

This unit describes the design, synthesis, purification, and amplification of a random-sequence DNA pool. Functional nucleic acid–binding or catalytic species can be selected from these random sequence pools. In designing the DNA pool, careful consideration should be given both to the degree of randomization and the length of the random sequence region (see Strategic Planning). Following pool design, chemical synthesis on a commercial DNA synthesizer will yield a single-stranded DNA pool. The newly synthesized oligonucleotide pool can then be purified (see Basic Protocol 1). Prior to amplification, the initial complexity of the pool should be determined (see Support Protocol 1), the skewing of the pool should be determined (see Support Protocol 2), and amplification reaction conditions should be optimized (Support Protocol 3). If the nascent synthetic oligonucleotide is judged to be suitable for large-scale amplification, it can be enzymatically converted into a double-stranded DNA library (see Basic Protocol 2). Multiple copies of a single-stranded DNA pool can be derived from each double-stranded DNA library, or the library can be transcribed to yield an RNA pool or a modified RNA pool (see *UNIT 24.3*). Figure 24.2.1 outlines the procedure.

STRATEGIC PLANNING

Designing the Initial DNA Pool

The nucleic acid pools used for in vitro selection experiments typically contain a randomized central core flanked by constant sequences that are required for enzymatic manipulations, such as PCR amplification, in vitro transcription, or restriction digestion (see also Fig. 24.2.2).

Since a pool is relatively expensive to synthesize, both in terms of time and cost, some effort should be devoted to pool design. There are many subtle parameters to consider that can greatly influence the outcome of a selection experiment, including the degree of randomization, pool length, and pool modularity (see Table 24.2.1 for references to

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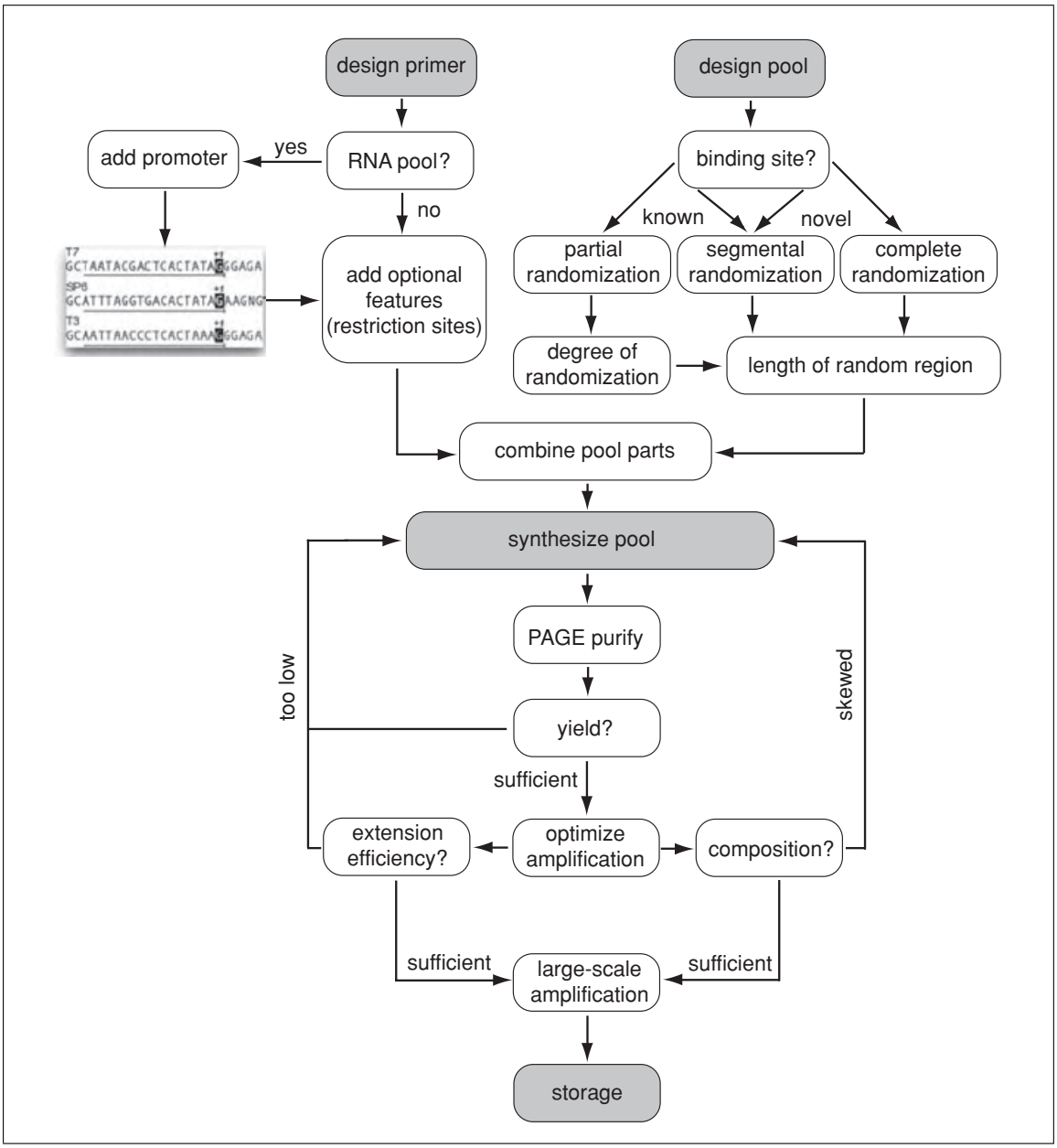

Figure 24.2.1 Flow chart outlining pool design, synthesis, and large-scale amplification.

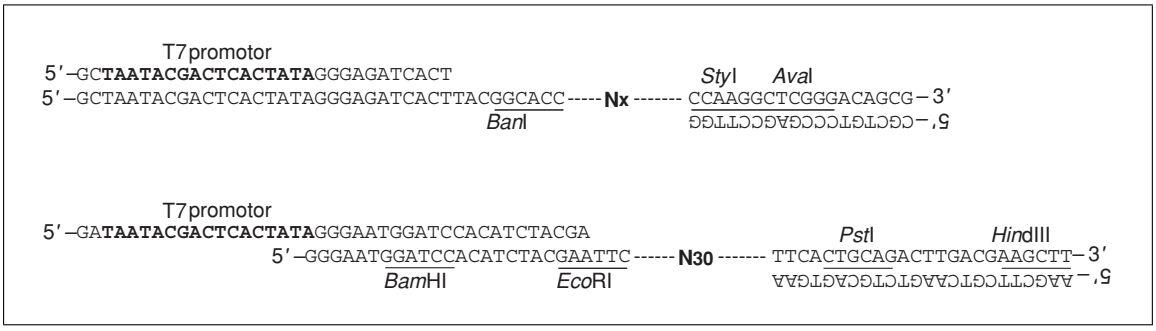

Figure 24.2.2 Two examples of pools used in in vitro selection. Primers are shown above and below the sequence of the pool. The T7 promoter is delineated in bold. Restriction sites are underlined, with their enzymes listed.

Table 24.2.1 Selection Experiments with Different Types and Sizes of Pools

Target	DNA/RNA	Length of random region	Reference
Bacteriophage T4 DNA polymerase	RNA	8	Tuerk and Gold (1990)
HIV-1 Rev	RNA	66, doped (65% wild type, 30% non-wild type, 5% deleted)	Bartel et al. (1991)
Ribozyme	RNA	120	Bartel and Szostak (1993)
HIV-1 Rev	RNA	30	Tuerk and MacDougal-Waugh (1993)
HIV-1 Rev	RNA	4 and 6, segmental; 6-9 and 6-9, segmental	Giver et al. (1993)
PKCβ	RNA	120	Conrad et al. (1994)
HTLV-1 Rex	RNA	43, doped (70% wild type, 30% non-wild type)	Baskerville et al. (1995)

selection experiments that have previously been successfully executed with different types and sizes of pools).

Type of selection and degree of randomization

Most researchers who carry out in vitro selection experiments wish either to better define or optimize a known binding site (binding-site selection), or to identify a novel binding site (aptamer selection). Each of these tasks requires the synthesis of different types of pools. The sequences and structures that contribute to known binding sites are frequently best defined by selections that start from partially randomized pools. One example of binding-site definition that started from a partially randomized pool was a selection that defined critical residues of the Rev-responsive element (RRE) of HIV-1 Rev (Bartel et al., 1991). This experiment is also described in more detail below. Biased pools can also be used for the optimization of a previously isolated motif. For example, aptamers that could bind to the Rex protein of HTLV-1 were selected from a partially randomized pool based on the wild-type Rex-binding element (XBE) but in the end bound Rex 9-fold better than the XBE (Baskerville et al., 1995). Doped sequence selections can also be used to better define the functional sequences and structures of aptamers obtained from completely random pools, as described below (Hessleberth et al., 2000). Doped sequence pools for aptamers typically retain from 70% to 95% sequence identity (5% to 30% mutation rate) in order to balance the population between the original, functional wild-type variant, large numbers of inactive sequences and structures, and a relatively small number of more active sequences and structures.

In contrast, completely random sequence pools explore a much wider swath of sequence space and are more useful for the isolation of novel binding species (aptamers) or catalytic species (Breaker, 1997; Jaeger, 1997). There are many examples of the selection of novel binding sites from completely random sequence pools (reviewed in Chandra and Gopinath, 2007, and Stoltenburg et al., 2007). Even when a natural binding site is known in advance, a completely different binding site may be selected from a random sequence pool; for example, Tuerk and MacDougal-Waugh (1993) isolated unique binders to Rev that bound better than the wild-type RBE sequence in vitro. Completely random sequence pools can also be used to extract aptamers that bind to proteins not normally thought to bind to nucleic acids; an example of this is the selection of an RNA aptamer that bound and inhibited the β isoform of protein kinase C (Conrad et al., 1994). Completely random sequence pools can also be used for the selection of novel nucleic acid catalysts.

For example, starting from a pool with a 220-position random region, Bartel and Szostak (1993) isolated a novel ribozyme capable of RNA ligation. Generally, selections for catalysis require pools with a random region greater than 90 residues, while binding selections use pools with a random region of less than 70 residues.

Intermediate between partially random and completely random sequence pools are segmentally random sequence pools. In a segmentally random pool, short tracts of sequence are completely randomized. Segmental randomization thus allows all possible sequences within a short region or set of residues to be examined. Thus, if a natural binding site is known, but a portion of that binding site is suspected to be particularly important for function, then a segmentally random pool can be used to identify all possible, functional sequences within the wild-type sequence context. For example, Tuerk and Gold (1990) selected aptamers that bound T4 DNA polymerase from a pool that contained 8 random sequence positions flanked by wild-type residues. Similarly, many binding sites are known to be presented within a particular structural context, such as a stem-loop or stem-bulge structure. In these cases, a portion of the structure can be completely randomized, and all possible functional stem-loops or stem-bulges can be identified. For example, the Rev-binding element was known to form a stem-internal loop-stem structure. Giver et al. (1993) segmentally randomized only the internal loop portion of the structure and selected Rev-binding species. Many of the anti-Rev aptamers had sequences that were significantly different than the wild-type, yet were still presented in the context of a stem-internal loop-stem structure.

Partially random (doped) pool design (binding site selection)

The most important issue in the synthesis of a doped pool is the level of randomization (the probability of sequence substitution/position). As a general rule, the substitution frequency of a doped pool should roughly correspond to the number of positions thought to be required for function. For example, if 10 residues within a nucleic acid binding site are thought to be functional, then the rate of substitution might be set to yield single mutants at least half the time. If the substitution frequency is set too low, there may be too few varying residues or combinations of residues to yield information about functional sequences or structures. In contrast, if the substitution frequency is set too high, the sequence space nearest the wild-type motif will only be sparsely sampled, and many of the highly mutated molecules may be nonfunctional because their sequences will have diverged too far from the wild-type.

For example, an in vitro genetic analysis has been used to uncover the critical structural interactions between the HIV-1 Rev protein and its primary RNA binding site, the Rev-binding element (Bartel et al., 1991). The RBE had previously been mapped by deletion analysis to a short segment of HIV-1. Bartel and his co-workers assumed that the minimal RBE was smaller even than the region identified by deletion analysis, and thus decided to heavily dope a portion of a 66-nucleotide sequence at a frequency of 35% substitution/position. The initial RRE library contained $\sim 10^{13}$ molecules that had an average of 23 substitutions/template (0.35 probability substitution/position $\times$ 66 positions $= \sim$23 substitutions); less than 1 in 10^{12} molecules were completely wild-type. Following selection, a 20-nucleotide core-binding site within the 66-nucleotide pool was readily defined by sequence conservations and co-varying residues. A lower substitution rate might not have precisely defined the relatively small binding site, while an even higher substitution rate might have created a mutational load that would have limited the selection of functional molecules or even have allowed the selection of novel, non-wild-type anti-Rev aptamers (Giver et al., 1993; Tuerk and MacDougal-Waugh, 1993). Conversely, if the binding site were larger than originally hypothesized, the relatively high rate of substitution might have meant that few functional molecules could have survived the selection unscathed.

The number and type of sequence substitutions, as opposed to the probable target size for mutation, can also be used to plan the synthesis of a doped sequence pool, as described by the following equations. Typically, a 1-μmol synthesis of a 100-residue template yields a pool of $\sim 10^{15}$ amplifiable molecules. Regardless of the degree of partial randomization or the precise doping strategy employed, the number of different mutational combinations is given by:

$$3^n\{L!/[n!(L-n)!]\}$$

where n is the number of sequence substitutions/template in a template of length L. For example, in the case of the 66-nucleotide RRE pool discussed earlier, there were $\sim 2.17 \times 10^9$ possible 5-residue substitutions and $\sim 1.25 \times 10^{16}$ possible 10-residue substitutions.

To calculate the fraction of a given set of substitutions that are actually contained in a doped pool, the binomial probability distribution can be used:

$$P(n,L,f) = \{L!/[n!(L-n)!]\}(f^n)(1-f)^{(L-n)}$$

where P is the fraction of the template population when f is the probability of substitution/position. If primarily single-base substitutions are desired, then f should be maximized for $n = 1$; if multiple mutations (e.g., double or triple substitutions) are desired, then f should be correspondingly higher. If the doping strategy is optimized for n substitutions, then this number of substitutions will occur most frequently, "$n-1$" and "$n+1$" substitutions will occur less frequently but in roughly equal numbers, and so forth. Higher levels of sequence substitution skew the mutant frequency distribution, allowing the sampling of some regions of sequence space to the exclusion of others (Fig. 24.2.3).

Therefore, in the RRE example already cited, a pool of 1×10^{13} molecules doped at a frequency of 35% would contain few 5-residue substitutions [$1 \times 10^{13} \times P(5,66,0.35) = \sim 1.82 \times 10^6$ 5-residue substitutions out of $\sim 2.17 \times 10^9$ possible 5-residue substitutions]. In contrast, if the pool were doped at a frequency of 18%, all 5-residue substitutions would almost certainly be included [$1 \times 10^{13} \times P(5,66,0.18) = \sim 9.3 \times 10^{10}$ 5-residue

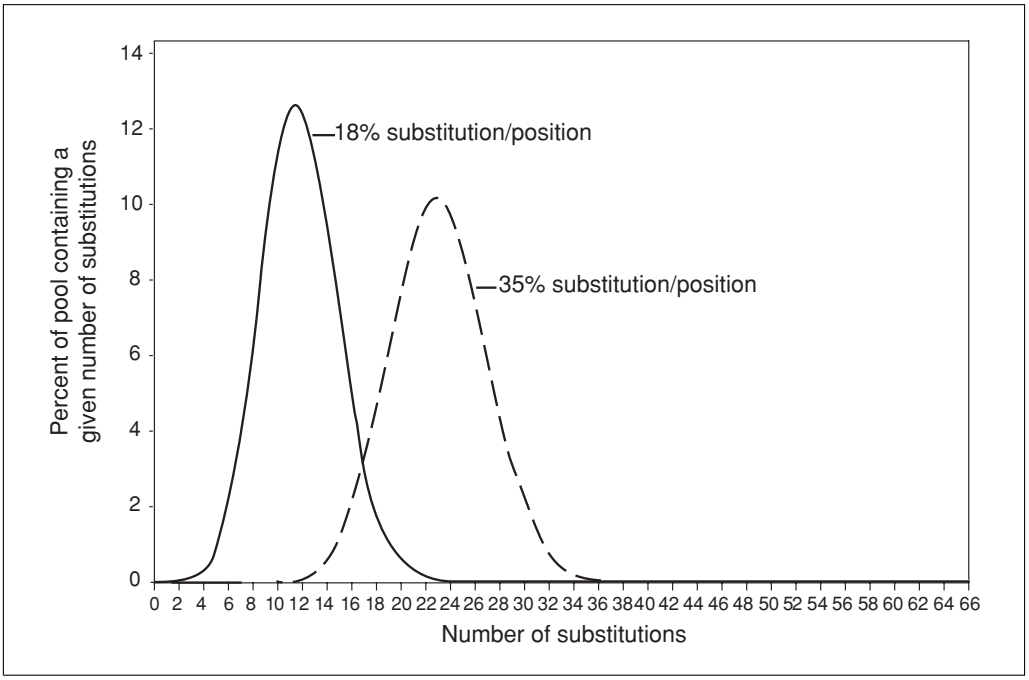
Percent of pool containing a given number of substitutions
0
2
4
6
8
10
12
14
18% substitution/position
35% substitution/position
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66
Number of substitutions

Figure 24.2.3 Comparison of substitution distributions for a 66-nucleotide pool doped to either 18% or 35%.

substitutions]. Note that in a pool of only 1×10^{13} total molecules, neither doping scheme would yield all possible 10-residue substitutions.

Completely random pool design (aptamer selection)

Completely random sequence pools are used to initiate selection experiments when no functional nucleic acid sequence or structural motif is known in advance. There is really only one parameter to consider when designing a completely random pool: the length of the random region. While this parameter is considered in detail below, we must first dismiss a frequent bogey of selection neophytes, the issue of complexity and representation.

Random sequence space is a vast landscape of possibilities of which only a vanishingly small fraction can be sampled by either nature or man. Assuming a 4-monomer repertoire from which pools can be constructed, there are $\sim 1.6 \times 10^{60}$ unique individual sequences in a sequence space bounded by a 100-residue template ($4^{100} = \sim 1.6 \times 10^{60}$), a quantity of nucleic acid greater than an Avogadro's number of Earth masses. While this grotesquely large value is clearly beyond the realm of experimental possibility, modern methods of chemical nucleic acid synthesis do allow the sampling of nearly as much sequence information as may be contained in the Earth's biosphere. As a back-of-the-envelope calculation, consider that there are on the order of $\sim 1 \times 10^9$ species in the biosphere, each with $\sim 1 \times 10^5$ genes. If each of these genes in turn is composed of $\sim 1 \times 10^3$ residues, then there are $\sim 1 \times 10^{17}$ residues worth of information in a biosphere. In contrast, a typical 1-μmol synthesis of a 100-residue random sequence pool would contain 1×10^{15} molecules $\times \sim 1 \times 10^2$ residues/molecule $= \sim 1 \times 10^{17}$ unique residues or roughly 1 biosphere's worth of information. Obviously, the connection and ordering of sequence information in organisms is important as well.

Typically, a 1-μmol scale column random sequence pool synthesis contains $\sim 1 \times 10^{15}$ molecules, and thus can potentially sample on the order of all possible 25-mers ($4^{15} = \sim 1.1 \times 10^{15}$). In fact, since different 25-mers can be found in different "reading frames," a slightly larger sequence space will likely be sampled. Because of this physical restriction, it is sometimes thought that random sequence pools should be no more than 25 residues in length—any longer, and only a fractional sampling would be possible, and many potential sequences would be lost. While this is true, it should be realized that longer pools do not lose any of the numerical complexity of smaller pools (except in those instances where long syntheses are extremely inefficient) and in fact gain access to some fraction of longer sequence and structural motifs as well. For example, tRNA molecules are roughly 76 nucleotides in length. It might prove more difficult to select tRNA mimics from a random sequence population containing 30 randomized residues than from a pool spanning 80 randomized residues. However, any short functional tRNA mimics present in the shorter population should also be present in equal or greater number in the longer population. In most instances, the relative completeness of the pool is not a consideration in the success of a selection. Indeed, it has been shown that functional nucleic acids are not extremely rare (for reviews see Gold et al., 1995, and Fitzwater and Polisky, 1996) and can be isolated both from "complete" pools that span 20 random sequence positions and from very "incomplete" pools that span 90 random sequence positions.

Having dismissed considerations of complexity and representation, the one guiding principle that emerges from this analysis is that longer pools are more generally useful for selection experiments than shorter pools. However, this principle must be applied with appropriate caveats. First, aptamers derived from shorter pools are easier to analyze. Sequence and structural motifs embedded within a 30-nucleotide random sequence region are much more readily apparent than sequence and structural motifs embedded within a 90-nucleotide random sequence region, especially if the motifs are not colinear. Second,

longer pools are more difficult and costly to synthesize than shorter pools. Finally, longer pools are more likely to yield amplification or other selection artifacts than shorter pools. For example, pools that contain random regions greater than 90 nucleotides in length can form self-aggregates that precipitate from solution upon prolonged incubation, and thus require immobilization on a solid support prior to selection (Bartel and Szostak, 1993; Lorsch and Szostak, 1994). Because of these considerations, pools used for the in vitro selection of aptamers typically contain from 20 to 80 random sequence positions.

Longer pools are not only desirable but are likely required in selections for complex functions, such as catalysis. Pools used for the selection of ribozymes typically contain from 50 to 220 random sequence positions (for recent reviews see Scott, 2007; Pan and Clawson, 2008; Piganeau, 2009). The optimal length of the random region is an active area of research (Sabeti et al., 1997) where many of the fundamental parameters remain to be defined. A computational analysis of structural diversity in RNA pools suggested that longer pools may not be substantially more functional than shorter pools (Kim et al., 2007), although our practical experience continues to suggest otherwise. Practically, though, longer pools must be synthesized as oligonucleotides of 150 residues or fewer in length because of the constraints of DNA synthetic chemistry. For this reason, pools longer than 150 bases are typically generated in a modular fashion by ligating together individual, synthetic oligonucleotides (Bartel and Szostak, 1993). Segments of shorter DNAs can be stitched together by the inclusion of unique restriction sites (Bartel and Szostak, 1993). Asymmetric restriction sites, such as *Ava*I (C|YCGRG), *Ban*I (G|GYRCC), and *Sty*I (C|CWWGG), where Y = C or T, R = A or G, and W = A to T, are very useful for this task since they minimize intra-pool dimerization via self-ligation. Also, these enzymes are cost-effective for digesting large amounts of DNA. Alternatively, an overlapping region can be included at the 3′ end of each synthetic oligonucleotide and mutually primed synthesis (e.g., *UNIT 8.2*) of a longer template can be carried out. After assembling pool modules, the complexity (yield) of the new, aggregate pool will need to be freshly assessed. The upper boundary of the complexity of an assembled pool (e.g., 10^{11} 100-mer modules $\times$ 10^{11} 100-mer modules) will likely be much larger than its actual complexity (e.g., 100 μg of ligated 200-mer, 9.12×10^{14} molecules).

Segmentally random pool design (binding site and aptamer selection)

In general, the rules governing the design of segmentally random pools are idiosyncratic, depending on experimental purpose. If the desire is to better define a known binding site, then relatively short sequence tracts (i.e., from four to ten residues) should be completely randomized. The randomization of longer sequence tracts may lead to the selection of novel binding sites rather than variants of a known binding site. The residues can either be colinear (as is the case for many DNA binding sites) or dispersed (as is the case for many RNA binding sites). If the desire is to identify a binding site within the context of a known structural element, then from four to twenty residues can be completely randomized. In this instance, the fewer the number of residues that are randomized, the more likely it will be that the selected sequences will resemble a wild-type binding site or retain an engineered structure. The greater the number of residues that are randomized, the more likely it will be that a novel aptamer sequence or structure will be discovered. Recently, computational models and simulations have been developed that might help in the design of "smart" pools (Chen, 2007).

Primer design

When designing pools, the constant sequences at the 5′ and 3′ ends of a pool function as primer-binding sites and can be almost any sequence or length. Primers of 20 nucleotides in length are convenient because their melting temperatures are convenient

for PCR and they can easily be synthesized in high yields. In designing constant sequences and complementary primers, obvious artifacts associated with the PCR, such as secondary-structure formation or self-association that could lead to the production of primer dimers, should be avoided. Web-based programs such as Integrated DNA Technologies' OligoAnalyzer (*http://www.idtdna.com/analyzer/Applications/OligoAnalyzer*) or MIT's PRIMER3 (*http://frodo.wi.mit.edu*) can assist in designing constant primer-binding regions. Each of these programs has initial variables that must be set. Utilizing the values that mimic reaction conditions (such as salt and dNTP concentrations in PCR) is suggested. As a rule of thumb, one should try to avoid using the same triplet sequence more than once in either of the constant regions; attempt to ensure that the GC content is between 45% and 60%; and check primer sequences to avoid self-dimerization, the formation of hairpins, and cross-hybridization (Singh and Kumar, 2001; Abd-Elsalam, 2003).

Beyond these basal considerations, there are two schools of thought regarding the sequence of the priming site itself. On the one hand, designing primers to possess a 3′ clamp of 5′-WSS-3′ (IUB codes: W = A or T, S = C or G), such as ACC, ensures good extension by polymerases. On the other hand, the inclusion of A/T-rich regions at the 3′ termini of primers reduces the frequency of mispriming and allows virtually "infinite" multiplication of DNA amplicons (Crameri and Stemmer, 1993). The inclusion of restriction sites within primer regions can facilitate cloning of selected nucleic acids into specific plasmids, although palindromes adjacent to the 3′ ends can also facilitate the genesis of primer-dimers. So-called T/A kits that take advantage of the propensity of *Taq* polymerase to incorporate untemplated adenines at the 3′ end of amplicons are also frequently utilized.

Finally, primers for partially randomized pools should be designed so that they do not conflict with the folding or accessibility of a known DNA or RNA binding site. It is suggested that the secondary structure of the wild-type binding site with any appended primer-binding sites be determined using an algorithm such as Mfold (Jaeger et al., 1989; Zuker, 2003). If the native or wild-type structure of the binding site is not among the most common folds, then the primers should be redesigned. Additional improvements in primer and probe design have been stimulated by the desire to carry out single nucleotide polymorphism analyses, whole-genome sequencing, phylogenetic analyses, and quantitative PCR (Vieux et al., 2002; Boutros et al., 2009). In addition, methods have begun to be developed that address the interference of constant sequences and primer binding sites during selection (Legiewicz et al., 2005; Pan and Clawson, 2009).

If an RNA pool is to be constructed, runoff RNA transcripts for in vitro selection are frequently made with T7 RNA polymerase. There are several known promoters for T7 RNA polymerase (Milligan et al., 1987), but the following minimal sequence gives good yields:

-17 -1

5′-TAA-TAC-GAC-TCA-CTA-TA-3′

Addition of a G and C residue at the -18 and -19 positions of the minimal promoter helps to close the DNA duplex and stabilize the 5′ end of the promoter region, thereby increasing transcriptional yields. Transcription initiation is optimal when there are stretches of purines in the +1 and +2 positions, with GG being the best initiator (Milligan et al., 1987). Transcriptional yields also increase if uridine does not appear in the transcript before position 6. Typical pool designs incorporating all the elements described are shown in Figure 24.2.2.

Chemically Synthesizing the Pool

While pools of genomic DNA sequences have been used for selection (Singer et al., 1997), partially or completely random sequence pools must be chemically synthesized. Modern DNA synthesizers utilize phosphoramidite chemistry (*UNIT 2.11*) or H-phosphonate chemistry (Strömberg and Stawinski, 2004) and can routinely produce usable amounts of DNA up to 150 nucleotides in length. Longer oligonucleotides can also be synthesized, but products of side reactions such as branching and depurination accumulate throughout the synthesis, and the amount of final, usable product recovered can be vanishingly small. Since stepwise coupling efficiencies for a long oligonucleotide are, on average $\geq$98%, the typical yield of a 100-base synthesis that starts with a 1-μmol column is 13.5%, or 13.5 nmol, or 1×10^{16} different molecules, of which $\sim$10% to 30% can be enzymatically elongated or amplified. Several strategies can be used to enhance the synthetic yield of oligonucleotides that are longer than 100 bases (see *UNIT 2.11*). Further, if a pool longer than $\sim$150 nucleotides is desired, smaller pools can be modularly synthesized and coupled by ligation or mutually-primed synthesis (see discussion of completely random pool design, above).

Deciding on commercial synthesis

With the advent of oligonucleotide synthesis companies such as IDT, Sigma, and Invitrogen, primers and pools can now be custom ordered. Because of reagent costs, the need for specialized synthetic expertise, and equipment overhead, it is frequently better to order a pool than to synthesize it in the lab. While the yield of homemade and outsourced pools is often similar, the quality of randomization and the overall synthetic integrity (number of extendable sequences) are typically much higher from synthesis companies (see Table 24.2.2 for a comparison).

In determining the costs for outsourcing, the length of the overall pool and the type of random region desired are the primary considerations. Many commercial supply houses with businesses focused on primer production set price ranges based on size, and thus longer pools are forced into higher price ranges. Most pools should be synthesized on either a 100 nmol scale (up to 90 nt) or a 250 nmol scale (90 to 100 nt). That said, there is a substantial difference in price between these two scales ($0.55 and $0.95 per nucleotide, respectively). There is also frequently a separate setup fee for mixing an "*N*" bottle of phosphoramidites.

The yield and quality of pools should also be considered when deciding between commercial and in house synthesis. In the authors' experience, yields were similar: for both a longer and shorter pool, around 10% of the synthesis could be recovered as full-length products (a coupling efficiency of $\sim$97.6 $\pm$ 0.2%; see Table 24.2.2). Pool complexity is also a function of the number of full-length sequences that can be replicated

Table 24.2.2 Comparison of Synthetic Methods for Two Pools

Pool	Synthesis method	Cost[a]	Crude yield	Coupling efficiency	Extension efficiency
N73	IDT[b]	$277.70	7.4%	97.7%	44%
N44	IDT[b]	$136.90	12.4%	97.5%	69%
N73	In house	$293	8.6%	97.8%	6%
N44	In house	$293	11.8%	97.4%	17%

[a]Costs reflect available discounts and are stated in 2009 dollars.
[b]IDT: Integrated DNA Technologies (*http://www.idtdna.com/*).

(extendability). In the authors' experience, commercial syntheses produces 4- to 7-times more replicable or extendable sequences than in-house syntheses. The overall randomness of pools is also a consideration. In the authors' experience, IDT does an adequate job of producing pools with little compositional skewing. When analyzing a sample of 17 variants from an N44 pool synthesized by IDT the base ratios of A:C:T:G were 25.4%:21.1%:25.9%:27.6% (744 total bases in the random region). In contrast, during in-house synthesis, coupling efficiencies of the different phosphoramidites must be painstakingly optimized to avoid skewing (as discussed below under In-house synthesis).

There are other trade-offs however, including the time of delivery. For in-house methods, pools can be synthesized, deprotected, and lyophilized in as little as one full day, while upwards of 2 weeks may be required for an outsourced order. In addition, when synthesizing pools in-house, additional syntheses do not greatly increase the cost, due to reagent quantities. Therefore, while synthesizing one pool in-house is often cost-prohibitive, synthesizing multiple pools may provide a savings over commercial sources.

In house synthesis

In certain cases, such as the production of doped pools, it may be desirable to perform a synthesis "in-house." IDT and other synthesis companies typically charge $100 for each hand-mixed bottle, and a doped pool utilizes five such bottles for doped regions that are less than or equal to 40 nt. For longer pools, the cost for doping may well be over $1000. Therefore, in-house synthesis of doped pools may still be the best option. Most synthesizers can be programmed for in-line, degenerate mixing of bases. While this method is useful when only a few positions must be randomized, because of the extremely fast reaction of the activated phosphoramidite with the newly deprotected 5′ hydroxyl, random sequences will be skewed towards the phosphoramidite that first enters the column. Therefore, for longer pools or pools that should contain a statistically random distribution of nucleotides, it is better to manually mix the phosphoramidites off-line and use this mixture for the synthesis of degenerate sequence positions. A more stochastic distribution can be obtained by including larger amounts of A and C phosphoramidites in the mix to compensate for the faster coupling times of G and T phosphoramidites (Zon et al., 1985). Suggested ratios include a 1.5:1.5:1.0:1.2 molar ratio of A:C:G:T phosphoramidites (D.P. Bartel, pers. comm.), a 1.30:1.25:1.45:1.00 molar ratio of A:C:G:T (Unrau and Bartel, 1998) and a 1.50:1.25:1.15:1.00 molar ratio of A:C:G:T (see User's Manual for PE Biosystems Models 392 and 394 DNA/RNA Synthesis).

Doped pools are among the most difficult to synthesize (Hermes et al., 1989; Bartel et al., 1991). Doping can be accomplished by using phosphoramidite mixtures that have been adjusted to ensure the proper level of partial randomization of a given nucleotide. For example, a 10% doped pool would contain 90% of the wild-type nucleotide at each doped position, and 3.3% of each of the non-wild-type nucleotides. If a doped pool is to be synthesized in which non-wild-type residues are included at a rate of 10%/position, then for the 2′-deoxyadenosine bottle, a molar ratio of 33.43:1.50:1.00:1.21 of A:C:G:T phosphoramidites should be used. These ratios were derived by first adjusting for the relative molecular mass and coupling differentials of the individual phosphoramidites, then mixing the phosphoramidite solutions on a percent volume basis to yield the desired extent of doping. This process is described in greater detail below.

To normalize the coupling of different phosphoramidites, relative correction factors that take into account different coupling efficiencies and molecular masses must be calculated. Multiplying together these correction factors gives an overall correction factor to provide equal molar coupling of each phosphoramidite. Table 24.2.3 displays

Table 24.2.3 Representative Calculations Based on the Masses and Efficiencies for Couplings that Utilize the Canonical Tetrazole Activation Chemistry and Phosphoramidites Bearing Standard Protecting Groups

Phosphoramidite[a]	Molecular mass (g/mol)	Mass correction	Coupling efficiency correction	Overall correction
5′-CE-dA	858	0.87	0.67	0.58
5′-CE-dC[b]	834	0.89	0.67	0.60
5′-CE-dG	840	0.89	1.00	0.89
5′-CE-dT	745	1.00	0.83	0.83

[a]CE (β-cyanoethyl)
[b]Ac-CE-dC can also be utilized for faster deprotection

Table 24.2.4 Volumes of Acetonitrile Needed to Dissolve 1 g of Phosphoramidite

Phosphoramidite	Volume acetonitrile (ml)
5′-CE-dA	11.6
5′-CE-dC	12.0
5′-CE-dG	17.8
5′-CE-dT	16.6

representative calculations based on the masses and efficiencies for couplings that utilize the canonical tetrazole activation chemistry (*UNIT 2.11* and Beaucage and Caruthers, 2000) and phosphoramidites bearing standard protecting groups [cyanoethyl for the phosphates along either isobutyryl (N-2 of guanine) or benzoyl (N-6 of adenine and N-4 of cytosine) groups]. Other chemistries and protections may require the substitution of other correction factors.

Most modern synthesizers require that ∼1 g of phosphoramidite be dissolved in ∼20 ml of acetonitrile to be used in the coupling reaction. Applying this constraint along with the combined mass-coupling (overall) correction factor gives the volumes shown in Table 24.2.4 to dissolve 1 g of each phosphoramidite. Therefore, if equal volumes of each of these solutions are mixed, equal molar coupling should occur since the molar concentrations have been adjusted to account for both the mass and coupling differentials. This bottle will be referred to as an equiactive "*N*" bottle.

To simplify the mixing of the four doped phosphoramidites bottles, it is customary to first resuspend each of the phosphoramidites in the corrected volumes of acetonitrile shown in Table 24.2.4. Equal volumes of these solutions are then mixed to create an equiactive "*N*" bottle. The doped bottles are then generated by mixing appropriate ratios of the equiactive "*N*" solution with individual phosphoramidites solution according to Table 24.2.5. As in the example above, if a 10% doped pool is to be synthesized in which non-wild-type residues are included, then, for each degenerate nucleotide, 1 volume of the equiactive "*N*" bottle should be mixed with 6.5 volumes of a given phosphoramidite.

In addition to varying nucleotide composition, it is also possible to vary the length of random sequence that is synthesized. Deletions can be stochastically incorporated during a synthesis by replacing the capping step with an acetonitrile wash (Bartel et al., 1991). It is more difficult to stochastically incorporate insertions, but the lengths of segmental random sequences in a pool can be mixed. For example, in Giver et al.

Table 24.2.5 Amidite Mixtures for a Given Level of Mutagenesis in a Doped Pool[a]

Equiactive phosphoramidite (volume ratio)[c]	Equiactive *N* (volume ratio)[b]								
	0.25	1	2	3	4	5	6	7	8
1	**15.0%**	37.5%	**50.0%**	56.3%	60.0%	62.5%	64.3%	65.6%	**66.7%**
2	8.3%	25.0%	37.5%	**45.0%**	50.0%	53.6%	56.3%	58.3%	**60.0%**
3	5.8%	18.8%	**30.0%**	37.5%	42.9%	46.9%	50.0%	52.5%	54.5%
3.5	**5.0%**	16.7%	27.3%	34.6%	40.0%	44.1%	47.4%	50.0%	52.2%
4	4.4%	15.0%	**25.0%**	32.1%	37.5%	41.7%	45.0%	47.7%	50.0%
5	3.6%	12.5%	21.4%	28.1%	**33.3%**	37.5%	40.9%	43.8%	46.2%
6	**3.0%**	10.7%	18.8%	25.0%	30.0%	34.1%	37.5%	40.4%	42.9%
6.5	2.8%	**10.0%**	17.6%	23.7%	28.6%	32.6%	36.0%	38.9%	41.4%
7	2.6%	9.4%	16.7%	22.5%	27.3%	31.3%	34.6%	37.5%	40.0%
8	2.3%	8.3%	15.0%	**20.5%**	25.0%	28.8%	32.1%	**35.0%**	37.5%

[a]Bold values represent common doping percentages per position.

[b]The equiactive "*N*" bottle should contain equal volumes of each of the resuspended phosphoramidites; see Table 24.2.4.

[c]Each of the phosphoramidites should be resuspended according to Table 24.2.4.

(1993), four columns were used to generate a pool with two random regions of 6 to 9 positions separated by a constant domain. The first column was synthesized with 6 random positions, the second with 7 random positions, etc. Following the addition of the intervening constant sequence, the synthesis was stopped, the four columns were opened, and the resins from the four columns were mixed. The mixed resins were then equally redivided into four new columns and the synthesis was resumed. The first column incorporated 6 positions, the second column 7 positions, etc. Thus, the first column contained oligonucleotides in which the first random segment was 6, 7, 8, or 9 residues long, and a second random segment that was uniformly 6 residues long. The second column contained oligonucleotides in which the first random segment was 6, 7, 8, or 9 residues long and a second random segment was uniformly 7 residues long, and so forth. Following the completion of all four syntheses, the reactions were combined to generate the final random sequence pool.

BASIC PROTOCOL 1

PURIFICATION OF A RANDOM SEQUENCE POOL

A newly synthesized oligonucleotide pool should be deprotected in accordance with the instructions provided for a given phosphoramidite reagent (see, for example, step 1, below), then lyophilized and purified on a denaturing polyacrylamide gel (*UNIT 2.12*) prior to amplification. Oligonucleotides can also be purified using HPLC or commercially available spin columns, but HPLC purification is not recommended for ssDNA pools due to concerns about cross-contamination. Since oligonucleotides of equivalent length but different sequence migrate at slightly varying rates (see User's Guide for PE Biosystems Expedite Nucleic Acid Synthesis System), a pool should appear as a broader band than a homogeneous sequence. In fact, because of the presence of capped failure sequences and depurinated, cleaved fragments, it is likely that the oligonucleotide product will appear even more heterogeneous. Failure sequences will include the mixture of products that are of the length $pool_{n-1}$, $pool_{n-2}$, $pool_{n-3}$, etc. Some of these foreshortened sequences can eventually be recovered by PCR.

As a general note, since sequences exist as single copies prior to amplification, individual species can be easily lost. Therefore, it is important to wash and elute the various filters,

tubes, and tips described below one or more times. The eluates can then be pooled for a final precipitation and eventual amplification.

Contamination of primers or other solutions with a synthesized or isolated pool should be avoided by using aerosol barrier tips. Similarly, gel plates used during purification should be washed thoroughly to ensure that they are free of contamination with other pools or primers.

Materials

DNA pool
Ammonium hydroxide
n-butanol
TE buffer, pH 8.0 (*APPENDIX 2*)
2× denaturing dye (see recipe)
3 M sodium acetate (*APPENDIX 2*)
Ethanol

Lyophilizer
75° and 90°C water baths
50-ml Sterile Conical Tube Filter Unit (Thermo Scientific Nalgene)
Fluorescent TLC plate (VWR), wrapped in plastic wrap
UV lamp
Razor blades
Small-bore syringes
13-ml centrifuge tubes capable of withstanding temperature extremes (Sarstedt)
Rotary shaker

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (e.g., *UNIT 2.12*)

1. After synthesis, deprotection, and cleavage from the solid support, lyophilize the oligonucleotide solution to dryness or precipitate with a 10-fold volume of *n*-butanol.

   For commercially synthesized pools, the nucleic acid has already been deprotected, cleaved, and desalted. Oftentimes, a commercial supplier will also provide the option to purify the pool via HPLC or PAGE.

   As an example, when utilizing Glen Research synthesis reagents, such as Sterling phosphoramidites and columns, the manufacturer suggests an 8-hr incubation at room temperature with 1 ml of ammonium hydroxide per 1 μmol synthesis for deprotection and cleavage. The resin is then washed with 3 volumes of diH_2O and lyophilized to dryness.

   The n-butanol precipitation can occur quite quickly at room temperature for longer oligonucleotides. Shorter (<20 base) oligonucleotides may require longer or colder incubations. To ensure more efficient recoveries of oligonucleotides it is safest to precipitate for ≥1 hr at −70°C.

2. Pour a 15 cm × 17 cm × 1.6 mm denaturing polyacrylamide gel (e.g., *UNIT 2.12*).

   To allow for good separation of near-full-length from non-full-length products, the acrylamide concentration should be chosen so that the full-length oligonucleotide will migrate approximately one-half to three-fourths of the way into the gel by the time the loading dye reaches the bottom.

   For a pool between 80 and 130 nt, this corresponds to an 8 to 10% gel. It is recommended that pools be sieved on a medium-format gel (15 cm × 17 cm) with 1.6 mm spacers to ensure good separation and to prevent overloading.

3. Resuspend the lyophilized or precipitated pellet in 100 μl of water or buffer (i.e., TE buffer, pH 8.0) per 250-nmol-scale synthesis, and add an equal volume of 2× denaturing dye. Heat denature samples at 75°C for 5 min prior to loading. Load the

entire 250 nmol scale synthesis or up to 1/3 of a 1 μmol synthesis per polymerized gel and perform electrophoretic separation (*UNIT 2.12*).

It is often convenient to load several (six) wells in the gel in parallel, although a single well that extends the breadth of the gel can also be loaded.

4. Place gel on a fluorescent TLC plate that has been wrapped in plastic wrap and excise the oligonucleotide product from the gel with the aid of a UV lamp, using razor blades.

   The desired oligonucleotide product is generally the darkest, shadowed band on the gel (excluding UV-absorbing material that runs at the dye front). If stepwise synthetic efficiency has been low, the product will appear as a smear instead of as a clear band. Since many of the n-1, n-2, etc. products can be converted into full-length products by the polymerase chain reaction, a fairly wide band of near full-length products can be cut from the gel. The excision should be carried out relatively quickly, since unnecessarily long UV exposure can damage the oligonucleotide product.

   The full-length oligonucleotide product should be the slowest-migrating band. However, if deprotection has been incomplete, lighter bands that migrate considerably above the major fully deprotected band may be observed.

   Unpolymerized acrylamide absorbs strongly at 211 nm and may cause shadowing at the edges and wells of the gel. This can obscure the resolution or recovery of bands in the outer lanes.

5. Elute the oligonucleotide from the gel slices as follows.

   a. To aid in the diffusion of the oligonucleotide from the acrylamide matrix, chop gel slabs into fine particles by forcing the gel through a small-bore syringe.

   b. Place the crushed gel slabs in a 13-ml centrifuge tube capable of withstanding temperature extremes.

   c. Add 3 ml of TE buffer, pH 8.0, per 0.5 ml of gel slab (typically corresponding to two wells). Do not exceed 13 ml of buffer for the entire gel slab. Place the sample at −80°C for 30 min or until it is frozen solid.

   d. Quickly thaw the tube in a hot water bath and then let it soak at 90°C for 5 min. Elute the DNA overnight at 37°C or room temperature on a rotary shaker.

      This freeze-rapid thaw approach (Chen and Ruffner, 1996) allows ice crystals to break apart the acrylamide matrix, increasing yield and decreasing elution time. Typically, 80% of a 20-mer oligonucleotide can be recovered after 3 hr of rotary shaking, making this technique comparable to electroelution (see, e.g., UNIT 2.7).

      Because elution is a diffusion-controlled process, higher elution volumes or serial elutions from the same gel slice can increase the amount of DNA recovered. Longer oligonucleotides diffuse from the gel more slowly than shorter sequences. Samples of especially long synthetic DNAs and RNAs that are particularly resistant to elution with aqueous buffers may be eluted more easily in 6 vol of formamide (>5 hr at room temperature), followed by a brief elution with an aqueous buffer (~1 hr). Isoamyl alcohol extraction (e.g., UNIT 2.12) can be used to bring the extracts to a convenient volume for subsequent precipitation.

6. Filter the eluted oligonucleotide through a conical tube vacuum filter unit to remove the remaining polyacrylamide gel fragments.

7. Precipitate the eluted oligonucleotide pool by adjusting the salt concentration to 0.3 M, adding from a 3 M sodium acetate stock solution, then adding 2.5 vol of ethanol. Keep at −20°C for 3 hr, then microcentrifuge at maximum speed, 4°C. Lyophilize to dryness. Resuspend the synthetic pool in TE buffer, pH 8.0 (to protect against nuclease contamination or drastic pH changes).

If the volume of the eluted oligonucleotide is too large to conveniently precipitate, concentrate the sample by extracting against an equal volume of n-butanol. Remove the upper butanol layer and repeat until the aqueous volume is convenient for precipitation. About 1/5 of the aqueous layer is extracted into the organic butanol layer for every volume of butanol used. If too much butanol is used, thereby completely extracting the aqueous layer into the butanol, add more water and repeat the concentration.

DETERMINING THE POOL COMPLEXITY

SUPPORT PROTOCOL 1

The number of different molecules present in a population can affect the outcome of a selection experiment (see Troubleshooting). If the pool complexity is too low for a given application, the pool will have to be resynthesized.

Pool complexity is, in turn, a function of yield and of the number of molecules in the pool that can be fully extended by a polymerase. The overall yield of the synthesis can be calculated by determining the UV absorption of the pool. However, deletions, incompletely deprotected residues, or backbone lesions that arise during chemical synthesis decrease by 10% to 40% the fraction of molecules in a synthetic pool that can be fully extended by polymerases. For example, the rate of insertions (presumably due to a DMTr group cleavage via tetrazole) has been measured to be as high as 0.4% per position, and the rate of deletions (presumably due to incomplete capping) has been found to be as high as 0.5% per position (A. Keefe and D. Wilson, pers. comm.). The number of usable DNA molecules that are actually present in a nascent pool can be calculated by determining the fraction of the pool that can be extended by *Taq* polymerase.

Materials

Purified ssDNA pool
PCR primers
T4 polynucleotide kinase and buffer (New England Biolabs)
[γ-^{32}P]ATP (>3000 Ci/mmol)
0.5 M EDTA, pH 8.0 (*APPENDIX 2*)
3 M sodium acetate (*APPENDIX 2*)
25:24:1 phenol/chloroform/isoamyl alcohol saturated with 10 mM Tris·Cl, pH 8.1/1 mM EDTA (see *UNIT 2.1A* or purchase from Sigma)
3.0 M sodium acetate
70% and 95% ethanol
TE buffer, pH 8.0 (*APPENDIX 2*)
1 mg/ml blue-dyed glycogen (GlycoBlue; Ambion)
10× PCR amplification buffer (see recipe)
Taq DNA polymerase
2× denaturing dye (see recipe)

Thermal cycler
15 cm × 17 cm × 0.75 mm denaturing polyacrylamide gel (*UNIT 2.12*)
Phosphor imager plate and phosphor imager (*APPENDIX 3A*)

Additional reagents and equipment for quantitation of DNA (e.g., *APPENDIX 3D*), end-labeling of DNA (e.g., *UNIT 3.10*), phenol/chloroform and chloroform extraction of DNA (*UNIT 2.1A*), PCR amplification (e.g., Chapter 15), denaturing polyacrylamide gel electrophoresis (*UNIT 2.12*), and phosphor imaging (*APPENDIX 3A*)

1. Quantitate DNA by UV absorption assuming that A_{260} of 1.0 indicates ~37 μg/ml of single stranded DNA.

   Also see APPENDIX 3D.

2. Label the 5′ end of the 3′ PCR primer with [γ-^{32}P]ATP by preparing the following reaction mixture:

   For a 20-μl reaction:
   2 μl 10× NEB T4 polynucleotide kinase buffer
   80 pmol dephosphorylated DNA, 5′ ends
   20 pmol (150 μCi) [γ-^{32}P]ATP
   10 U T4 polynucleotide kinase

   Incubate 60 min at 37°C, then stop the reaction by adding 1 μl of 0.5 M EDTA. Phenol/chloroform and chloroform extract the labeled oligonucleotide (see *UNIT 2.1A*), and precipitate by adding one-tenth volume of 3 M sodium acetate (for a final concentration of 0.3 M) and 2.5 volumes of 95% ethanol to precipitate the RNA. Mix and incubate at −80°C for 15 min. Microcentrifuge 10 to 15 min at maximum speed, 4°C, to recover the precipitate. Wash the pellet with cold 70% ethanol and dry the pellet completely. Redissolve the labeled DNA pellet in 20 μl of TE buffer, pH 8.0.

   Also see UNIT 3.10.

   The authors frequently include 3 μl of a 1 mg/ml blue-dyed glycogen solution to increase the yield of nucleic acid precipitation and to better visualize the pellet. If glycogen would prevent binding to a given target, transfer RNA can also be used as a carrier, but will obfuscate the quantification of the pool RNA (see below).

   The primer concentration after this step should be 4 μM. The volume of the reaction and the concentration of DNA and [γ-^{32}P]ATP will vary depending on application.

   This procedure ensures that most of the unincorporated label remains in the supernatant. In addition, a desalting column can be employed to ensure complete removal of unincorporated label prior to the phenol/chloroform extraction.

3. In two separate reactions, incubate ∼10 pmol of labeled primer with (or without) a 10-fold molar excess of pool in a 30-μl extension reaction in 1× PCR amplification buffer, under the same conditions that will be used in the final amplification, in a thermal cycler as follows (see, e.g., *UNIT 15.1* for PCR).

   a. Denature and anneal the primer and template DNA in 1× PCR amplification buffer.

      Typical thermal cycling conditions include denaturation at 94°C for 5 min, annealing at ∼50°C for 1 min, and extension at 72°C for 20 min. More commonly referred to as a Taq extension assay, this procedure is one cycle of PCR with a long extension step.

   b. Finally, terminate the reaction by the addition of an equal volume of 2× denaturing dye.

4. Heat the extension reaction to 90°C for 3 min and load the reaction on a 15 cm × 17 cm × 0.75 mm denaturing polyacrylamide gel. Electrophorese until the dye is at or near the bottom of the gel, but do not let the radiolabeled primers run off.

   Also see UNIT 2.12.

   It is also useful to load a separate well with an aliquot of the primer alone to verify that the band is of the correct size. Appropriately radiolabeled size markers can also be used to gauge size. Choose an acrylamide percentage that allows efficient separation of small primers from larger extended products.

5. Dry and expose the gel to a phosphor imager plate. Using a phosphor imager (*APPENDIX 3A*), quantify the control primer band and the extended product band (see Fig. 24.2.4 for expected results).

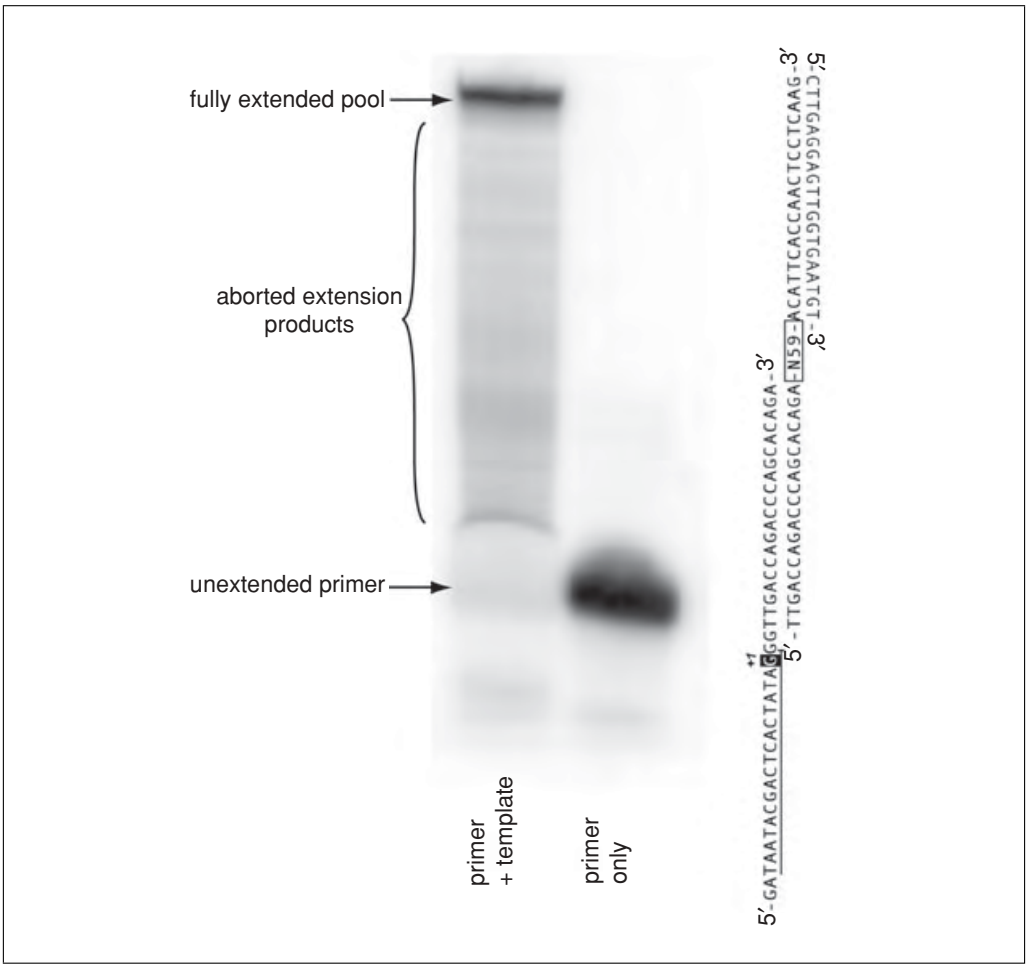

Figure 24.2.4 Typical extension reaction. The pool used (N59) is shown to the right, next to the figure of the gel. Lane 1 shows the fully extended product and a large number of extensions on incomplete or damaged templates. Lane 2 is a control reaction containing only the primer. The extension reaction was incubated for 30 min.

There may be a smear leading up to the extended band. Determining how much near-full-length material to include in the quantitation is a somewhat subjective decision. Calculate the percent extension by dividing counts of labeled, extended product by counts of labeled primer. Percent extension for a gel-purified ssDNA pool can range from 10% to 30% for in-house syntheses to as high as 75% for commercial syntheses. The complexity of the pool is then the yield (determined in step 1) multiplied by the extension efficiency (percent extension determined above). If the complexity of the pool is insufficient for planned experiments, then the pool must be resynthesized.

DETERMINING THE POOL BIAS

SUPPORT PROTOCOL 2

Following extension, the reaction should be repeated using a cold primer and the non-radioactive double-stranded DNA pool should be amplified in a PCR reaction, cloned (e.g., using a TA cloning kit from Invitrogen), and individual members sequenced to determine the degree of randomness. The cloning step could also be carried out following PCR optimization (see Support Protocol 3). From 20 to 30 clones should be sequenced to determine the base composition of the starting pool. The random region should be composed of roughly 25% of each base. A pool with the random region skewed toward one or more bases (>30%) should be resynthesized.

SUPPORT PROTOCOL 3

SMALL-SCALE PCR OPTIMIZATION OF POOL AMPLIFICATION

To enhance yield and further avoid bias, the amplification conditions for a pool should be optimized prior to carrying out a large-scale amplification. Moreover, since amplifying a pool is costly in terms of both time and money, any optimization of the PCR should first take place on a small scale. The more involved large-scale amplification can then be carried out with confidence.

Materials

Purified ssDNA pool
PCR primers
PCR amplification buffer (see recipe) containing 1.5 mM Mg^{2+}
dNTP mix (dATP, dCTP, dGTP, dTTP; *UNIT 3.4*)
Taq DNA polymerase (e.g., New England Biolabs)
3.8% NuSieve 3:1 agarose gel (Cambrex; also see *UNIT 2.5*)
1× TBE buffer (*APPENDIX 2*)
dsDNA mass markers (e.g., Invitrogen)

Thermal cycler
Densitometer

Additional reagents and equipment for PCR (Chapter 15) and agarose gel electrophoresis (e.g., *UNIT 2.5*)

1. Carry out a 100-µl PCR reaction using a 1:50 dilution of synthetic pool oligonucleotide as template, 2 µM primers, and PCR buffer with 1.5 mM magnesium. Use the manufacturer's suggested quantity of *Taq* polymerase (e.g., 2.5 U of New England Biolabs *Taq*) in a reaction containing 200 µM each dNTP. A suggested temperature regime is:

1 cycle:	5 min	95°C	(denaturation)
	1 min	50°C	(annealing)
	20 min	72°C	(extension)
1 to 10 additional cycles:	30 sec	95°C	(denaturation)
	1 min	55°C	(annealing)
	1 min	72°C	(extension).

After 4 to 8 cycles of amplification, check the length and purity of the amplified DNA on a 3.8% Nu Sieve agarose gel in 1× TBE buffer (e.g., *UNIT 2.5*) using dsDNA mass markers.

Conditions for the initial extension step should mimic those in step 3 of Support Protocol 1 to maintain pool complexity. The annealing step should be modified to reflect predicted primer melting temperatures and conditions.

Annealing temperature may need to be adjusted to as low as 45°C depending on primer composition (e.g., for a small or AU-rich primer). A gradient PCR can be carried out to assay different annealing temperatures simultaneously and thereby optimize the amplification procedure (see Fig. 24.2.5 for expected results).

A 100-µl reaction typically yields ~1 µg, but the amount can vary from 0.1 to 10 µg. A fuzzy band may indicate that too many cycles of PCR have been carried out. In this case, set up the reaction again and perform fewer cycles.

2. Dilute the double-stranded PCR DNA product 1:128, and repeat the PCR reaction, removing a 5- to 10-µl aliquot during the last 10 sec of the cycle-7 extension step.

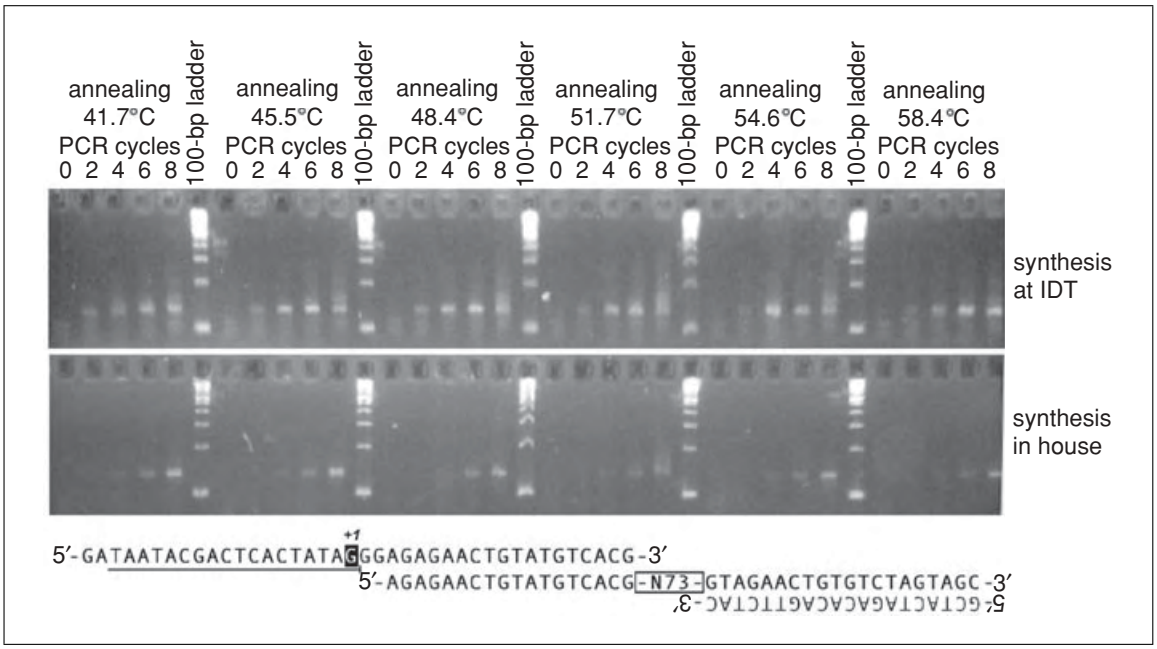
annealing 41.7°C PCR cycles 0 2 4 6 8
100-bp ladder
annealing 45.5°C PCR cycles 0 2 4 6 8
100-bp ladder
annealing 48.4°C PCR cycles 0 2 4 6 8
100-bp ladder
annealing 51.7°C PCR cycles 0 2 4 6 8
100-bp ladder
annealing 54.6°C PCR cycles 0 2 4 6 8
100-bp ladder
annealing 58.4°C PCR cycles 0 2 4 6 8
synthesis at IDT
synthesis in house
+1
5′-GATAATACGACTCACTATAGGGAGAGAACTGTATGTCACG-3′
5′-AGAGAACTGTATGTCACG-N73-GTAGAACTGTGTCTAGTAGC-3′

Figure 24.2.5 A PCR cycle course and optimization of annealing temperature. The gel follows amplification of the N73 pool across a gradient of annealing temperatures. Two different pool synthesis methods were analyzed. Samples were removed after 0, 2, 4, 6 and 8, cycles. The pool used in the cycle course is depicted below the figure of the gel. IDT: Integrated DNA Technologies (*http://www.idtdna.com/*).

Serially dilute the amplified product 1:2, 1:4, . . . 1:128. Electrophorese all of the samples on a large agarose gel (*UNIT 2.5*).

Note that it is quite difficult to accurately pipet solutions at 72°C. It may therefore be desirable to pipet an amount slightly larger than that intended for use in the serial dilution.

3. Calculate the average PCR amplification efficiency by identifying to what extent the cycle-7 PCR reaction is the result of progressive doublings of the original synthetic DNA. Determine which dilution lanes lack detectable DNA.

   The largest dilution that lacks detectable DNA is also the dilution that is a minimum estimate of the number of doublings. For example, if the 1/64 dilution is the largest dilution without detectable DNA, this implies that six "doublings" of the synthetic DNA yielded at least 64-fold more DNA. This is expressed as follows:

   $$(\text{average efficiency})^{\text{no. of theoretical doublings (i.e., PCR cycles)}} = \text{fold increase in DNA}$$

   Thus, if 7 cycles of PCR were performed, then the average number of doublings per cycle is ~1.81 [from $(\sim 1.81)^7 = 64$].

4. Modulate PCR conditions to enhance PCR efficiency.

   If the pool's average number of doublings per cycle is <1.8, then the PCR conditions chosen may skew the representation of the pool. In that case, PCR conditions should be modulated to enhance PCR efficiency. The following parameters or variables are most amenable to modification. It is best to begin the optimization with a single set of reaction conditions, modify individual parameters relative to this one reference reaction, and then combine all advantageous alterations into a single reaction. For more information on PCR see UNIT 15.1.

   Theoretically PCR can proceed until the primers or dNTPs are depleted. Therefore, primer and dNTP concentrations should be well above those used for the amplification of small amounts of DNA. Primer concentrations from 1 μM to as high as 5 μM have been used (although concentrations >5 μM are generally not helpful). It may be useful to scan both above and below 2.5 μM in 0.5-μM increments.

Magnesium concentration affects both primer annealing and the fidelity of Taq (which decreases with increasing magnesium concentration). Starting at the magnesium concentration supplied in the PCR buffer (usually 1.5 mM), scan in 1-mM increments toward 5 mM as a maximal concentration.

DNA denaturation at temperatures above 95°C is usually impractical, since this greatly reduces Taq's half-life. While other thermostable polymerases can be more resistant to higher temperatures, they usually have a lower extension efficiency and are more expensive than Taq. Annealing temperatures are dependent upon both primer sequence and length. The primer annealing temperatures should already be known from the primer design process, or may be calculated via an algorithm that can be found at http://idtdna.com/analyzer/Applications/OligoAnalyzer/. This algorithm takes into account nucleotide composition, stacking energies (according to Turner's rules), and empirical data. An annealing temperature ~5°C less than the calculated annealing temperature is a good place to begin optimization. The amplification is more efficient at a lower annealing temperature, but mispriming and secondary-structure problems are more pronounced. Higher temperatures improve the specificity, but decrease the overall yield of the reaction. To determine the optimum annealing temperature for a given primer and magnesium concentration, one should scan in both directions around the annealing temperature in 5°C increments. Finally, extension temperatures are modulated by the properties of Taq, which will extend (although inefficiently) at temperatures as low as 65°C. When extending at temperatures above Taq's optimum temperature (70° to 75°C), somewhat more polymerase may be required; scanning of the enzyme quantity should be done in 2.5-U increments. However, too much Taq may be harmful to structured single-stranded nucleic acids (Lyamichev et al., 1993).

5. Confirm the results of the extension reaction described in Support Protocol 1 by the optimization method as follows. After optimizing pool PCR conditions for >1.8 average number of doublings per cycle, determine the pool complexity by performing another 0.1-ml PCR reaction with 2 nM of the original, synthetic pool oligonucleotide under the now optimized reaction conditions. After 7 or more cycles of PCR, perform agarose gel electrophoresis on serial dilutions of the PCR reaction adjacent to serial dilutions of dsDNA mass markers. Calculate the amount of amplified DNA either using a densitometer or by estimating which dilutions are most similar. Calculate the approximate pool complexity as follows:

$$\frac{\text{g of PCR DNA after } N \text{ cycles of PCR}}{\text{g avg. no. of doublings per cycle (see step 4)}} = \text{g of starting extendable ssDNA}$$

$$\frac{\text{g of starting extendable ssDNA}}{330\ \text{g/mol} \times (\text{no. of bases in full-length product})} = \text{mol of starting extendable ssDNA}$$

$$\text{mol of starting extendable ssDNA} \times (6.02 \times 10^{23}) = \text{molecules of starting extendable ssDNA}$$

$$\frac{\text{molecules of starting extendable ssDNA}}{\text{starting molecules}} = \text{fraction of extendable ssDNA}$$

$$\text{fraction of extendable ssDNA} \times \text{no. of synthetic pool molecules} = \text{pool complexity}$$

PCR efficiency should be optimized to balance the average number of doublings per cycle against the total reaction volume. A pool of 1×10^{15} molecules ($\sim 1.7 \times 10^{9}$ mol) at a starting template concentration of 2 nM will require 0.85 liters for amplification. Therefore, it is greatly desirable to amplify the pool at the highest template concentration that still gives a reasonable number of doublings per cycle. The amplification should generate at least 8 copies of pool DNA if the pool complexity is to be archived and preserved (see Basic Protocol 2).

LARGE-SCALE PCR AMPLIFICATION OF POOL DNA

BASIC PROTOCOL 2

Very long and complex pools often require PCR amplification on a multiple-milliliter scale. Large-scale PCR differs from conventional PCR in that it is typically conducted in water baths using 15-ml, 17 × 120-mm, screw-capped (Sarstedt) thermostable tubes to accommodate the larger volumes. Amplification reactions of up to 2.5 liters have been carried out in this way. Medium-scale amplifications can sometimes be carried out in thermal cyclers that can accommodate multiple samples (e.g., 96-well PCR plates).

Materials

Purified ssDNA pool and primers
0.5 M EDTA, pH 8.0 (*APPENDIX 2*)
2-butanol (for larger volumes)
3 M sodium acetate
Ethanol
TE buffer, pH 8.0 (*APPENDIX 2*), containing 50 mM of a salt such as KCl

Thermal cycler or three water baths (one must be a circulating water bath)
96-well PCR plate or 13-ml thermostable tubes (Sarstedt)
Thermometer
Styrofoam racks
Spectrophotometer or fluorimeter

Additional reagents and equipment for PCR amplification (*UNIT 15.1*; see Support Protocol 3 for determination of conditions on a small scale) and phenol/chloroform and chloroform extraction of DNA (*UNIT 2.1A*)

Plan the reaction

Since large-scale reactions are quite expensive in terms of nucleotides and enzyme, preparedness and planning for the large-scale amplification cannot be overemphasized. Primers <20 bases in length usually do not need to be gel purified and can instead be purified by precipitation.

1. After identifying the optimal PCR conditions on a small scale (see Support Protocol 3), prepare reagents for the large-scale reaction. Set aside time for the large-scale amplification, which will probably consume an entire day.

   The size of the large-scale reaction will be determined in part by the amount of DNA pool to be amplified and by the number of copies of the library that are desired. For example, one copy of a dsDNA pool with a complexity of 1×10^{15} weighs $\sim$100 μg. Assuming a 16-fold amplification in which the typical amount of DNA recovered from a 100-μl PCR reaction is 1 μg, then each 100-μl reaction should have 1 μg/16 = 60 ng of DNA. 100 μg total/60 ng/100 μl = 1667 × 100 μl, or a 167-ml reaction.

   The authors normally start with a complexity to 1×10^{14} sequences and carry out a 10-fold amplification. These parameters are ideally suited for one to two 96-well PCR plates that will be inoculated with 20 to 50 μl (total) of the pool. Actual amounts will of course depend on synthetic yield, extension efficiency, and amplification efficiency.

Choose how the amplification will be carried out

If the volume of the large-scale amplification reaction is to be ≤100 ml

2a. Use a commercially available thermal cycler repetitively. Set the reaction mixture up in advance, and pipet 100-μl aliquots into individual wells of a 96-well PCR plate.

3a. Carry out several small amplification reactions in advance to ensure that the optimized conditions determined in Support Protocol 3 work with the PCR plate format, and that amplification is uniform across the PCR plate.

4a. Perform thermal cycling on the entire reaction using multiple PCR plates.

For larger volumes

Reactions will be divided into aliquots in 13-ml thermostable (Sarstedt) tubes and amplified in a series of water baths. Construct floating racks by cutting off the bottom of the tubes' Styrofoam packing material. Reinforce these racks by wrapping their edges with heavy tape. Place the racks iteratively in three circulating or static water baths held at the denaturation, annealing, and elongation temperatures previously determined (see Support Protocol 3).

2b. Determine how long it will take for the reaction mixture in a tube to come to thermal equilibrium by constructing a temperature probe, placing a thermometer through the top of a 13-ml Sarstedt tube filled with 10 ml of water. Place the probe in a rack with other, similar tubes.

Typical equilibration times range from 2 to 8 min, depending on the temperature differential. Annealing and extension times of 5, 6, and 7 min are typical. It should be noted that these ramping temperature profiles are very slow relative to a commercial PCR machine and can yield more amplification artifacts.

3b. To ensure that the reaction conditions actually work as planned, fill the rack with tubes of water, a single amplification reaction, and the temperature probe. Denature the sample for 30 min, and then add *Taq* after the first annealing step. Take aliquots at each cycle to monitor the progress of the reaction.

4b. When reaction conditions have been confirmed, proceed with the remaining amplification reactions. Allow the final extension step to proceed for at least 20 min to ensure that all templates are completely double-stranded.

Do not be alarmed if the solution becomes cloudy; the detergent in the buffer causes the turbidity.

Amplification efficiencies of 3 to 4 doublings in 5 cycles can typically be achieved using this method.

5. Following the amplification, pool the reactions from the individual wells or tubes. Chelate the magnesium in the buffer by adding 1.1 molar equivalents of EDTA, pH 8.0 (from 0.5 M stock).

The reactions can be left at 4°C overnight.

6a. *If the PCR reaction volume is ≤100 ml:* Proceed directly to step 7.

6b. *If the PCR reaction volume is ≥100 ml:* Add an equal volume of 2-butanol and extract to concentrate the reaction to a manageable volume (usually 10- to 20-fold). Mix the layers by vortexing and then separate by centrifuging 5 min at 1200 × *g* at room temperature, then discard the upper, 2-butanol layer. Repeat as necessary.

About one-fifth of the aqueous layer is extracted into the organic 2-butanol layer for each volume of butanol used.

7. After concentrating the DNA, carry out a phenol/chloroform extraction, followed by two successive chloroform extractions (see *UNIT 2.1A*).

   At this point, it should be possible to easily precipitate the DNA. Be sure to temporarily save all of the organic layers in case of a mishap. Falcon tubes (50 ml) work well for these extractions, as they are conveniently sized and have a small surface area. Alternatively, a Teflon extraction funnel may be useful since nucleic acids will not stick to its surface.

8. Precipitate the DNA by adding one-tenth volume of 3 M sodium acetate (final concentration, 0.3 M) and 2.5 vol ethanol in 13-ml Sarstedt tubes if possible.

   If larger tubes are required, prepare a set of Beckman 250-ml high-speed centrifugation bottles. Wash the centrifugation bottles with 15 ml of 3% hydrogen peroxide for 30 min and then rinse three times, each time with 100 ml of distilled water to remove any residual DNases that may remain from previous use (typically bacterial cell pelleting).

9. Resuspend the amplified DNA in 100 to 200 µl TE buffer, pH 8.0, containing 50 mM of a salt such as KCl.

   It is unwise to resuspend a double-stranded DNA pool in water, since the random segments may denature, reassort, and become transcriptionally incompetent.

   If it is suspected that the pool has become denatured (for example, if a large single-stranded DNA component is seen on a nondenaturing agarose gel), simply repeat one to two cycles of PCR.

10. Quantitate the PCR DNA.

    Determine the overall amplification efficiency and the final number of DNA molecules. This can be done by carrying out gel electrophoresis in parallel with dilutions of a DNA ladder of known concentration. The concentration can also be determined spectrophotometrically or by monitoring the change in absorbance of an intercalated fluorescent dye, Hoechst 33258 (Sigma), on a fluorimeter (e.g., DyNA Quant 200, GE Healthcare). These latter methods are much more quantitative (although the fluorimeter method may not be accurate for sequences <100 nucleotides in length). However, these methods may not distinguish precipitated double-stranded DNA from residual, precipitated nucleotides or single-stranded primers.

    The amount of DNA obtained from large-scale amplification is often referred to in terms of the number of copies of the original synthetic pool's complexity. For example, if the starting pool had a complexity of 1×10^{15} molecules and 8×10^{15} total DNA molecules were recovered, then, on average, 8 copies of the original starting pool were obtained from the amplification. It should be noted that skewing may arise during amplification. In addition, statistical skewing will occur during sampling of the amplified pool and may cause this estimation to be inaccurate; nevertheless, it is empirically useful.

11. Following large-scale amplification, store at least 4 copies of the pool at −80°C.

    Because of the aforementioned sampling errors, archiving at least 4 copies worth of the pool DNA ensures the preservation of most of the pool's complexity. The amount of preserved pool complexity can be calculated using the following equation:

    % of the pool complexity in a given sample $= 100 \times \{1\text{-}[(x - y)/x]^x\}$

    where x is total number of pool copies, and y is the number of pool copies archived.

    Therefore, in the example given above, if 4 of the 8 copies of the pool generated through amplification are archived, then ~99.6% of the original starting pool's complexity is preserved. Similarly, at least 4 copies of the pool should be used whenever manipulations such as ligation, transcription, or biotinylation, are carried out, so that the original complexity is also manifest in the manipulated or synthesized copies.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Denaturing dye, 2×

TBE buffer (*APPENDIX 2*) containing:
0.1% (w/v) bromphenol blue
7 M urea
Store up to 6 months at –20°C

PCR amplification buffer, 10×

500 mM KCl
100 mM Tris·Cl, pH 8.3 (*APPENDIX 2*)
x mM $MgCl_2$
0.1% (w/v) gelatin
Store in aliquots at –20°C

This solution can be sterilized by autoclaving. Alternatively, it can be made from sterile water and stock solutions, and the sterilization omitted.

15 mM $MgCl_2$ in the 10× buffer is the concentration (x) used for most PCR reactions. However, the optimal concentration depends on the sequence and primer of interest and may have to be determined experimentally.

COMMENTARY

Background Information

As early as 1955, researchers began developing methods to chemically synthesize oligonucleotides (Michelson and Todd, 1955). Modern synthetic procedures utilizing phosphoramidite chemistry and solid phase supports were developed and refined during the 1970s and early 1980s (Beaucage and Caruthers, 1981). The synthetic procedure has been reviewed extensively (Beaucage and Iyer, 1992; Brown, 1993; Iyer and Beaucage, 1999; Reese, 2005). Current oligonucleotide synthetic methods involve a stepwise addition of nucleoside phosphoramidites to the 5′-hydroxyl of an oligonucleotide immobilized on Controlled Pore Glass (CPG) resin. The dimethoxytrityl (DMTr) protecting group of the oligonucleotide is first de-blocked with trichloroacetic acid (TCA). This step produces a free trityl that can be monitored spectrophotometrically to assess extension efficiency. Then, the phosphoramidite is activated by tetrazole, and nucleophilic attack of the free 5′-hydroxyl results in the formation of a phosphite bond. This process is very fast (<30 sec) and typically goes to near completion (97% to 100%). Uncoupled oligonucleotides are capped with acetic anhydride and 1-methylimidazole to prevent further elongation. The capped sequences account for reduced yields during longer syntheses. During the last step of the synthesis cycle, the phosphite bond is oxidized with iodine and pyridine to yield the more familiar phosphotriester. The DMTr group on the newly incorporated phosphoramidite is then deprotected, and the cycle starts over. Variations on this cycle allow for the incorporation of phosphorothioates, unnatural C-5′-C3′ linkages, and other, more chemically challenging nucleosides.

With the development of de novo oligonucleotide synthesis, it became possible to not only carry out site-specific mutagenesis but also to create random sequence pools. Hermes et al. (1989) used "spiked oligos" to select for second-site suppressor mutations that could rescue the catalytic activity of triosephosphate isomerase, while Oliphant and Struhl (1989) carried out similar selections with β-lactamase. It also became apparent that functional nucleic acids could be selected from random sequence pools, and the Struhl lab also selected double-stranded oligonucleotide binding sites for the yeast DNA-binding protein GCN4 (Oliphant et al., 1990). This work set the stage for many of the directed evolution experiments that are carried out to this day.

Critical Parameters

Synthesis

Depending on the size of the pool to be synthesized, the operation of the DNA synthesizer may first need to be optimized. Short

pools (<80 total nucleotides in length) can be synthesized using standard protocols (see, e.g., PerSeptive Biosystems, 1998). In order to synthesize longer pools (>80 total nucleotides in length), all reagents should be fresh, and special care should be taken to exclude water from the synthesis (see *UNIT 2.1A*). To ensure equimolar base incorporation in the random region of longer pools, the phosphoramidites must be mixed in a skewed ratio (see Strategic Planning). Coupling efficiency should be monitored throughout the synthesis by following the trityl cation output (see *UNIT 2.11*).

Amplification

Optimization of PCR conditions according to established protocols is vital to the success of the large-scale amplification. Cycle temperatures and times, as well as the concentrations of polymerase, primers, and dNTPs (see, e.g., *UNIT 15.1*), should be addressed prior to the large-scale workup. Most importantly, since extremely large quantities of relatively expensive reagents (e.g., *Taq* polymerase) may be required, care should be taken to make sure that all reagents and procedures are in readiness. Different priming sequences often require distinct PCR buffers for optimal extension efficiency; the best buffer for a given pool and primer combination can be easily and systematically identified through the use of a PCR optimization kit (e.g., the PCR Optimizer Kit from Invitrogen).

Troubleshooting

The most common problem with the synthesis of a random sequence pool is the overall synthetic yield. However, researchers should carefully decide how many sequences are really necessary for their selection experiments. In selection experiments from a pool with a relatively limited potential diversity (i.e., a segmentally random pool with only 1×10^{11} possible sequences or less), even a low synthetic yield should be sufficient. However, in vitro selection from a pool with a very high potential diversity (i.e., a completely random pool with 1×10^{15} possible sequences or more) should use at least 1×10^{14} different sequences initially in order to adequately sample the potential sequence space. Pools that contain fewer than 1×10^{13} possible sequences should not be used.

The most likely sources of low yields and coupling efficiencies are old (i.e., water-contaminated) synthesis reagents. Thus, instead of attempting to amplify an incomplete pool, the pool should be resynthesized with fresh reagents; the old and new pools can then be combined, if desired. If fresh synthesis reagents do not significantly raise yields, then more serious problems, such as line or valve blockage, may be the cause, and the instrument service representative should be contacted.

The second most common problem is that the base composition of a partially or completely random region is skewed. Unfortunately, skewing cannot be detected until after completion of a large-scale amplification. Fortunately, unless the degree of skewing is extreme, it should not seriously affect the outcome of a selection. Moreover, if the degree of skewing is known in advance of a selection, it can be taken into account when analyzing the results of the selection. For example, Baskerville et al. (1995) selected functional Rex-binding elements from a partially randomized pool. Despite the fact that the initial pool did not contain equimolar representation of non-wild-type bases at partially randomized positions, these authors were able to determine the relative importance of individual residues by comparing the degree of conservation or variance before and after selection. If a researcher decides that extant skewing of base ratios is unacceptable, this can only be fixed by adjustment of the randomized phosphoramidite mixture and resynthesis of the pool.

The third most common problem is that the pool fails to efficiently elongate. With the proviso that the efficiency of extension may be as low as 10% of the available pool, it should not be much lower (i.e., 1% of the available pool). If extension or PCR efficiency is dauntingly low, the PCR conditions should be reexamined and optimized as described, including buffer and enzyme concentrations, temperatures, and extension times. Switching to a different thermostable polymerase, or to a combination of polymerases, will sometimes improve primer extension. If all possible PCR optimization conditions have been addressed, poor extension efficiency could reflect a problem with the synthetic DNA. For example, the pool may not have been completely deprotected or a primer binding site may have become largely depurinated during the course of a long synthesis. Although incomplete deprotection is rarely a problem, small aliquots of the pool can be further treated with ammonia, and extension and amplification can again be assessed. If additional deprotection instead yields oligonucleotide degradation, then it is likely that apurinic sites have accumulated, and the pool will have to be resynthesized.

Anticipated Results

It is apparent from the discussion earlier in this unit that there is no one correct way to design and amplify a random sequence pool (Piasecki et al., 2009). However, by following the protocols described above, results similar to the following should be observed.

If the integrity of the nascent, synthetic pool is good, then the primer extension efficiency (described in Support Protocol 1) should be relatively high. Figure 24.2.4 shows a typical extension reaction for a pool synthesized in the authors' laboratory. Molecules that were incapable of full extension make up the smear leading to the full-length product. By determining the number of counts in the full-length product relative to the radiolabeled primer, the extension efficiency for the pool was calculated to be ~39%.

Assuming that the nascent pool is intact and can serve as a template for the primer extension reaction, then it should be possible to amplify the pool via PCR. Figure 24.2.5 shows the results of an amplification "cycle course" for a different pool (N73, with a 73-nucleotide random sequence core). A 10-ml PCR reaction was aliquotted into multiple 96-well PCR plates and cycled on a BioRad DNA Engine thermocycler. The samples in the figure were withdrawn at 0, 2, 4, 6, and 8 cycles.

Time Considerations

The amount of time required for the protocols described in this unit should not be underestimated. Pool design will take at least 1 day, depending on the degree of background research required. It is strongly recommended that pool design be discussed with one or more colleagues prior to synthesis. The synthesis of oligonucleotides <150 bases in length can be easily accomplished in 1 day, allowing 1 hr to ensure proper instrument setup. Commercial synthesis companies are frequently almost as fast, but in some cases may take up to two weeks to deliver the pool. Pool purification and optimization of PCR conditions should take 1 to 2 additional weeks. Finally, the actual large-scale amplification and subsequent isolation of the dsDNA pool will require the researcher's undivided attention for ~2 days.

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In Vitro Selection of RNA Aptamers to a Protein Target by Filter Immobilization

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ABSTRACT

This unit describes the selection of aptamers from a pool of single-stranded RNA by binding to a protein target. Aptamers generated from this selection experiment can potentially act as protein function inhibitors, and may find applications as therapeutic or diagnostic reagents. A pool of dsDNA is used to generate an ssRNA pool, which is mixed with the protein target. Bound complexes are separated from unbound reagents by filtration, and the RNA:protein complexes are amplified by a combination of reverse transcription, PCR, and in vitro transcription. *Curr. Protoc. Mol. Biol.* 88:24.3.1-24.3.27.
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Keywords: aptamer • in vitro selection • affinity reagent • filter binding assay • SELEX

INTRODUCTION

An aptamer is a selected nucleic acid binding species. Typically aptamers are selected from random sequence pools, and form three-dimensional structures with binding pockets comparable to those formed by proteins. While there are multiple ways that aptamers can be selected in vitro (for current reviews, see Chandra and Gopinath, 2007; Kulbachinskiy, 2007; Stoltenburg et al., 2007), this unit will describe one of the most common: selection of aptamers that bind to a protein target from a single-stranded RNA pool. Aptamers generated from these types of selection experiments can potentially function as protein inhibitors, and may find applications as therapeutic or diagnostic reagents. In short, a double-stranded DNA pool (see *UNIT 24.2*) will be transcribed to generate a single-stranded RNA pool (Basic Protocol 1 in this unit). The initial concentration of protein target to be used is determined by labeling an aliquot of the pool (see Support Protocol 1) and performing the binding assay as described in Support Protocol 2. Following purification, the pool is mixed with the protein target. Binding species are separated from nonbinding species by nitrocellulose filtration (see Basic Protocol 2). RNA:protein complexes are then eluted from the filter, and binding species are amplified by a combination of reverse transcription, the polymerase chain reaction (PCR), and in vitro transcription (see Basic Protocol 3). The progress of the selection will be monitored by assaying the affinity of the radiolabeled RNA pool for the protein target after several rounds of selection (see Support Protocol 3). These steps are then repeated until a significant increase in binding is observed or until the diversity of the pool has been completely plumbed. The procedure is summarized in Figure 24.3.1.

TRANSCRIPTION AND ISOLATION OF RNA POOLS

BASIC PROTOCOL 1

The following protocol describes the preparation of the RNA pool to be used for selection. Starting from the dsDNA pool, the RNA is transcribed and purified by denaturing polyacrylamide gel electrophoresis. Recovery of the RNA from the gel is followed by ethanol precipitation of the RNA. Additional instructions can be found in *UNIT 3.8*. The directions

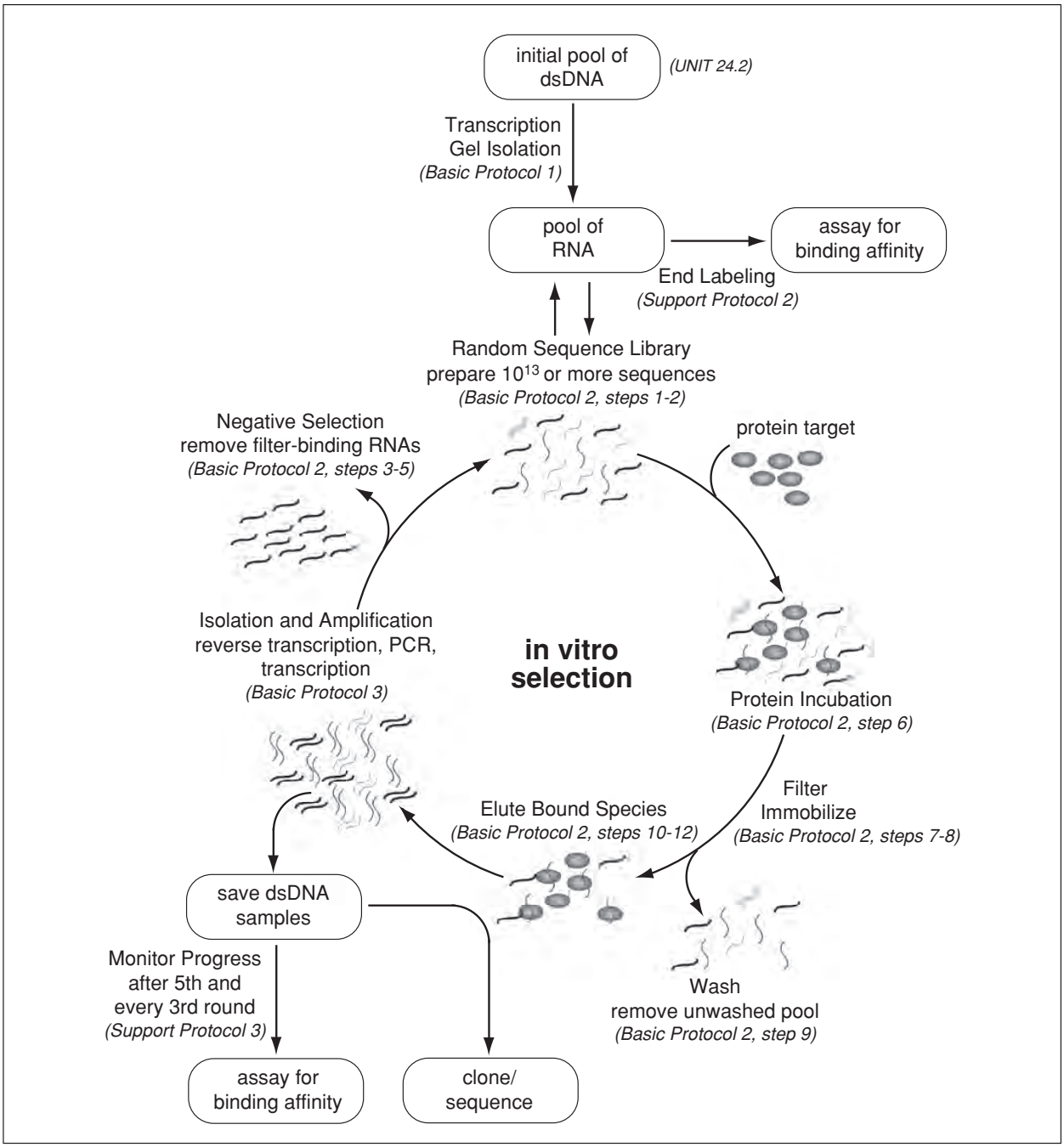

Figure 24.3.1 Steps involved in in vitro selection of RNA aptamers.

provided here are specific for the isolation of nucleic acid pools. As is the case for the original amplification of DNA pools (*UNIT 24.2*), many of the procedures described here can potentially lead to the cross-contamination of different RNA selection experiments or different generations of the same selection experiment. To avoid cross-contamination, it is wise to always use barrier tips, and to use disposable plastic Pasteur pipets rather than automatic micropipettors for large-volume transfers.

Materials

Double-stranded DNA pool (*UNIT 24.2*)
High Yield AmpliScribe T7 In Vitro Transcription Kit (Epicentre)
8% polyacrylamide denaturing gel (see recipe and *UNIT 2.12*)
2× denaturing dye (see recipe)

TBE buffer (*APPENDIX 2*)
TE buffer, pH 8.0 (see recipe)
3 M sodium acetate (*APPENDIX 2*)
70% and 95% ethanol

Thermal cycler, incubators, or heat blocks set at 37° or 42°C (for transcription) and 65° to 75°C (for denaturation)
UV light source
Fluorescent TLC plate (VWR) wrapped in plastic wrap
Sharp razor blade, fresh or thoroughly cleaned
Spectrophotometer, such as a NanoDrop (Thermo Scientific)

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (*UNIT 2.12*)

NOTE: All solutions and buffers should be made with deionized water (18.2 MΩ resistivity), filtered through a 0.2-μm polyethersulfone (PES) membrane, and sterilized by autoclaving. Use sterile, disposable plasticware and micropipet tips with filter barriers where possible. See Chapter 4 introduction and *UNIT 4.1* for guidelines on standard methods to protect against contaminating RNases. If ribonuclease contamination is found to occur, it may be eliminated by treating water with diethylpyrocarbonate (DEPC; see *UNIT 4.1*).

Perform initial round of transcription

Use the double-stranded DNA pool generated in *UNIT 24.2* (which should contain a T7 RNA polymerase promoter) as a template for in vitro transcription with T7 RNA polymerase.

1. Following the protocol provided with the kit, add ~1 μg of double-stranded DNA template generated as in *UNIT 24.2* to the transcription mix for a 20-μl total reaction volume. Incubate reaction at 42°C for 4 hr or overnight at 37°C.

   Depending on the length and initial complexity of the pool, 1 μg of double-stranded DNA will represent ~10^{13} different sequences while 10 μg represents ~10^{14} different sequences. The dsDNA concentration from the large-scale PCR (UNIT 24.2) should be determined by electrophoresis on an agarose gel and compared with a quantitation standard. The initial quantity of dsDNA used for selection should be calculated based on a desired number of starting species or total pool complexity (also see UNIT 24.2). It should be kept in mind that the overall complexity of the unamplified pool and the extent of amplification must be known in order to carry out these calculations (also see UNIT 24.2). The authors typically seed the transcription with 1 to 3 copies of the amount of dsDNA pool corresponding to the desired complexity.

   The AmpliScribe High Yield T7 kit can be used to produce between 20 and 100 μg RNA from 0.5 to 1 μg starting dsDNA. This yield equates to between 40 and 200 copies of each sequence originally present. The kit can be used with up to 8 μl of dsDNA template in a 20-μl reaction. If more RNA is desired for initial or subsequent rounds of selection, a proportionately larger transcription reaction should be attempted.

   In some instances it will be desirable to radiolabel the RNA. For example, it is relatively easy to determine whether and how much RNA binds to a filter in the presence or absence of a protein target by radiolabeling the initial pool (Support Protocol 1). An [α-^{32}P]nucleoside triphosphate—e.g., 0.5 μl [α-^{32}P]GTP (GE Healthcare Life Sciences) in a 20-μl total volume—can be included in the reaction mixture in addition to all the other reagents. Varying the proportion of "hot" to "cold" nucleoside triphosphates can control the specific activity of the RNA pool. Since the overall yield of the transcription reaction will generally be important, the specific activity of the nucleoside triphosphate mixture should be varied by increasing the amount of radioactive nucleotide added, rather than by decreasing the amount of unlabeled nucleotide present. Again, commercial transcription kits can be obtained that are geared towards the incorporation of labeled nucleoside triphosphates (RiboScribe, Epicentre).

2. In order to remove DNA from the transcription reaction, after the transcription incubation has been completed, add 1 μl of RNase-free DNase I from the Epicentre kit per 20-μl reaction and incubate for 25 min at 37°C.

   Because individual members of the double-stranded DNA library can potentially bind nonspecifically to either the target or to the selection matrix and subsequently be amplified, the DNA template should be removed from the transcription reaction according to this step, prior to proceeding with the selection. The effectiveness of this step can be evaluated by PCR analysis of reverse transcription in the absence of reverse transcriptase.

   It is essential that RNase-free DNase, such as that provided with the kit, be used; otherwise contaminating ribonucleases may destroy the newly transcribed RNA. An alternative would be to add RNase inhibitors to impure DNases, but such inhibitors themselves frequently contain endogenous ribonucleases that can be released during the incubation.

Purify the RNA pool

The RNA pool should generally be purified by denaturing gel electrophoresis.

3. Prepare a 0.75-mm thick, denaturing 8% acrylamide gel (see Reagents and Solutions and, e.g., *UNIT 2.12*).

   An 8% acrylamide concentration is convenient for the purification of RNA molecules from 60 to 150 nucleotides in length. However, the concentration of acrylamide used to separate the full-length transcript from incomplete transcripts is ultimately contingent upon the size of the RNA and should be chosen so that the RNA will migrate approximately half-way through the gel when the loading dye has reached the bottom (see UNIT 2.12).

   If the RNA sample contains a significant amount of nascent structure (for example, a doped sequence population that is based on a tightly folded secondary structure), it may not fully denature. Thus, it may be advisable to warm the gel to ~55°C by first pre-running the gel at a higher voltage (300 to 400 V). The temperature of the gel can be monitored using adherent thermometers (VWR).

   In some cases, very large amounts of RNA may need to be purified (for example, the initial transcription of an extremely complex DNA library may yield upwards of a milligram or more of an RNA library). In these instances, it may be desirable to purify the RNA library by either gel-filtration or ion-exchange chromatography (e.g., Qiagen RNA kit). However, the purification of the initial or subsequent pools should never be neglected, as foreshortened amplicons can arise and overtake selected populations.

4. Fully denature the RNA pool by adding an equal volume of 2× denaturing dye, and heat the RNA-dye mix for 3 min at 65° to 75°C.

   Although each species in the pool has a different sequence and shape, they should migrate similarly when fully elongated.

   Using a higher temperature or longer denaturing time risks hydrolysis of the RNA into smaller fragments, given the high concentration of Mg^{2+} present in the transcription buffer.

5. Thoroughly rinse each well of the gel prepared in step 3 with TBE buffer using a plastic Pasteur pipet, 1000-μl micropipettor tip, or syringe prior to loading (to remove urea, which will otherwise leach into the wells and form a barrier between the loaded sample and the gel). Load samples directly on the gel (a single 20-μl transcription reaction will typically fit into a 1-cm-wide lane). Run electrophoresis for 45 min to 1 hr at 400 V, until the bromphenol blue dye front reaches the bottom of the gel.

   If the wells are not cleaned prior to loading, the resolution of the separation can be compromised, especially if large amounts of RNA are being isolated.

6. Visualize the RNA bands by UV shadowing on a fluorescent TLC plate covered with plastic wrap, then excise the bands. Be sure to cut with a sharp razor blade and cut only the shadowed regions that contain the bulk of the RNA.

   There may be extra bands in the lane that correspond to incomplete transcripts or undigested DNA. The use of a size standard in a neighboring lane is recommended. Note, however, that the size standard should not itself be amplifiable, as cross-contamination of a single sequence with the RNA pool would drastically skew the distribution of sequences in the purified pool. Similarly, the razor blade used for excision should not have come into contact with other potentially amplifiable sequences, and should either be fresh or be cleaned extensively. Finally, if multiple selections are being carried out in parallel, they should be separated by at least two wells, or on a different gel entirely.

7. Immerse the gel slices in 1× TE buffer, pH 8.0, at ∼1 ml buffer/cm^2 of gel (typically, slices from three lanes) and incubate at 37°C overnight with agitation to elute the RNA pool.

   The TE buffer is necessary to inhibit trace quantities of ribonucleases.

   For quicker elution, use a 1-ml syringe plunger to crush the gel chunks into a slurry in a 1.7-ml microcentrifuge tube. Resuspend the slurry in 400 μl of 1× TE buffer, then incubate the slurry at −80°C for 10 min to use ice crystals to fully break up the acrylamide matrix. Elute the ssRNA from the gel at 65° to 75°C for 15 min. Repeat the elution with an additional 400 μl TE buffer.

   The authors routinely recover 95% of the nucleic acid with this procedure. To increase recovery, additional elutions can be performed, but increased incubation at elevated temperatures increases cleavage of RNA molecules.

8. Decant the eluate with a micropipettor and 1000-μl tip to separate the RNA-containing supernatant from the gel slice. Filter the elution through a 0.45-μm nitrocellulose membrane (such as Millipore Ultrafree-MC microcentrifuge filter tube) to remove acrylamide fragments.

Precipitate and quantitate the RNA

9. Add one-tenth volume of 3 M sodium acetate for a final concentration of 0.3 M and 2.5 volumes of 95% ethanol to precipitate the RNA. Mix, then incubate at −80°C for 15 min. Microcentrifuge 10 to 15 min at maximum speed, 4°C, to recover the precipitate.

   The authors frequently include 3 μl of a 1 mg/ml blue-dyed glycogen solution (GlycoBlue, Ambion) to increase the yield of nucleic acid precipitate and to better visualize the pellet. If the selection target binds to or interacts with glycogen, then this step should be omitted. Transfer RNA can also be used as a carrier, but will obfuscate the quantification of the pool RNA (see below).

10. Wash the RNA pellet with cold 70% ethanol and allow the pellet to dry completely.

    The pellet can be air dried, dried under a nitrogen or argon stream, or dried in a SpeedVac evaporator. The first method is least likely to result in cross-contamination of nucleic acid species; the last method is least likely to lead to degradation. In any event, keep the tube covered with Parafilm to avoid inadvertent nuclease contamination (poke holes in the Parafilm with a sterile pipet tip to allow evaporation to occur).

    If the RNA pool is particularly short (≤50 nucleotides), use cold 95% ethanol for the wash step.

11. Resuspend the RNA pellet in 25 μl TE buffer, pH 8.0.

    To avoid disturbing the composition of the selection buffer, the pellet can also be resuspended in RNase-free water. The small amount of EDTA present in TE buffer, however, will limit ribonuclease degradation of the pool, since ribonucleases frequently require a divalent metal to function. In some instances (e.g., small-volume PCR reactions), the presence of EDTA may have to be compensated for by adding more magnesium to the reaction.

12. Estimate the quantity of the RNA spectrophotometrically by measuring the absorbance at 260 nm.

Use an extinction coefficient of 0.025 ml/cm·μg (see, e.g., APPENDIX 3D). In practical terms, measure the A_{260} of a 1:10 dilution of the sample on a NanoDrop spectrophotometer or cuvette-based spectrophotometer. The A_{260}/A_{280} and A_{260}/A_{230} ratio should be between 1.8 and 2.2. If ratios are outside of these ranges, the purity of the original RNA sample may be suspect (with residual acrylamide or salt being the most likely contaminants), and the sample should be reprecipitated prior to use.

SUPPORT PROTOCOL 1

RADIOLABELING RNA FOR USE IN AN INITIAL AFFINITY ASSAY

Radioactive RNA can be generated either by incorporation of an [α-^{32}P]nucleoside triphosphate during transcription or by transfer of the terminal phosphate of γ-^{32}P ATP to the 5′ terminus of a dephosphorylated RNA molecule. The authors tend to prefer the latter method, despite the additional labor involved in preparation, because the specific activity of the sample is higher, less RNA is required for assays, and dissociation constants are correspondingly easier to compute.

Materials

RNA pool (Basic Protocol 1)
10× alkaline phosphatase buffer (New England Biolabs)
Calf alkaline phosphatase (New England Biolabs)
25:24:1 phenol/chloroform/isoamyl alcohol saturated with 10 mM Tris·Cl, pH 8.0/1 mM EDTA (*UNIT 2.1A*)
Chloroform
3 M sodium acetate (*APPENDIX 2*)
70% and 95% ethanol
1 mg/ml blue-dyed glycogen (GlycoBlue; Ambion)
10× PNK buffer (New England Biolabs)
10 U/μl T4 polynucleotide kinase (PNK; New England Biolabs)
167 mCi/ml [γ-^{32}P]ATP (7000 Ci/mmol; ICN Biomedical or GE Healthcare Life Sciences)
42° and 75°C water baths
Centri-Sep Spin Columns (Princeton Separations)

NOTE: All solutions and buffers should be made with deionized water (18.2 MΩ resistivity), filtered through 0.2-μm polyethersulfone (PES) membrane, and sterilized by autoclaving. Use sterile, disposable plasticware and micropipet tips with filter barriers where possible. See Chapter 4 introduction and *UNIT 4.1* for guidelines on standard methods to protect against contaminating RNases. If ribonuclease contamination is found to occur, it may be eliminated by treating water with diethylpyrocarbonate (DEPC; see *UNIT 4.1*).

Dephosphorylate the 5′ triphosphate termini of the isolated RNA pool

1. Mix the following components:

1 μg RNA in <3.5 μl volume
0.5 μl 10× alkaline phosphatase buffer
1 μl (1 U) calf alkaline phosphatase
x μl RNase-free water for a total reaction volume of 5 μl.

The RNA sample may need to be reprecipitated to obtain an adequately concentrated sample. If so, the precipitate can be resuspended directly in the reaction buffer or mixture.

Calf alkaline phosphatase is preferred over bacterial alkaline phosphatase because the activity can be heat-killed (see step 4) prior to the addition of the radiolabel.

2. Incubate at 42°C for 20 min to 2 hr.
3. Add 95 μl RNase-free water.
4. Heat-denature the calf alkaline phosphatase for 10 min at 75°C.
5. Perform a phenol/chloroform extraction (see Basic Protocol 2, steps 13 and 14).

   If the sample will be gel-isolated, this step can be omitted. If the radiolabeled sample will merely be precipitated prior to use, this step should be included.

6. Ethanol precipitate the RNA by adding one-tenth volume of 3 M sodium acetate (0.3 M final), 3 μl of 1 mg/ml blue-dyed glycogen, and 2.5 volumes of 95% ethanol, microcentrifuging, and washing the pellet with 70% ethanol (see Basic Protocol 1, steps 9 and 10). Allow pellet to dry completely.

   Avoid precipitating RNA in the presence of ammonium acetate, since ammonium ions inhibit the T4 polynucleotide kinase used in the next step.

7. Resuspend the dried pellet in a minimal volume (3 to 10 μl) of RNase-free water.

Perform kinase reaction

8. Set up the kinase reaction as follows:

   0.5 to 3 μl dephosphorylated RNA pool (from step 7)
   0.5 μl 10× PNK buffer
   1 μl (10 U) T4 polynucleotide kinase (PNK)
   0.5 μl (83 μCi) [γ-^{32}P]ATP (7000 Ci/mmol)
   x μl RNase-free H_2O for a total volume of 5 μl.

   Only a very small amount of RNA will be used in the binding assay (~50 pM in a 100 μl reaction). Unless multiple experiments are contemplated, the specific activity of the sample can be kept quite high by using a very small amount of RNA in the kinase reaction.

9. Incubate for 1 hr at 37°C.

   During this step, it is helpful to hydrate the Centri-Sep desalting columns.

10. Heat-inactivate the kinase in the reaction mixture at 70°C for 10 min, and increase the volume to 20 μl with water.
11. Apply the diluted kinase reaction directly to the middle of the Centri-Sep gel bed and centrifuge 2 min at 450 × g, room temperature, collecting the flowthrough.
12. Perform a phenol/chloroform extraction (see Basic Protocol 2, steps 13 and 14).
13. Ethanol precipitate the RNA by adding one-tenth volume of 3 M sodium acetate (0.3 M final), 3 μl of 1 mg/ml blue-dyed glycogen, and 2.5 volumes of 95% ethanol, microcentrifuging, and washing the pellet with 70% ethanol (see Basic Protocol 1, steps 9 and 10). Allow pellet to dry completely.
14. *Optional:* To fully purify the radiolabeled RNA pool, isolate the transcript by polyacrylamide gel electrophoresis as described in Basic Protocol 1, steps 3 to 8.

    If this is done, the phenol/chloroform extractions and the final precipitation of the RNA (steps 12 to 13 of this protocol) can be omitted. While unincorporated, radioactive triphosphates can also be removed by gel electrophoresis, the authors recommend utilizing the desalting (Centri-Sep) column to limit opportunities for radioactive contamination.

    The chief disadvantages of gel isolation are the time required for sample preparation and the relatively low efficiency of recovery of the radiolabeled RNA pool. However, since only a small amount of RNA pool is required for the binding assay, such low yields can frequently be tolerated. The authors frequently gel isolate radiolabeled RNA pools to ensure the integrity of RNA samples prior to carrying out binding assays.

SUPPORT PROTOCOL 2

BINDING ASSAY WITH THE END-LABELED RNA POOL TO DETERMINE THE OPTIMAL PROTEIN CONCENTRATION FOR SELECTION

To determine the initial concentration of a protein target to be used in a selection experiment, it is necessary to measure the affinity of the unselected pool for the protein target. The aggregate dissociation constant of the pool:protein complex can be calculated by determining the fraction of radioactively labeled RNA that can be bound at various protein concentrations.

The radiolabeled RNA is incubated in the binding buffer and protein solutions are added. The binding reaction is filtered through a vacuum manifold containing nitrocellulose and nylon membranes, and the fraction of RNA bound to the target is calculated to obtain a value for the dissociation constant. The nitrocellulose membrane will capture RNA:protein complexes, while the nylon membrane will capture all free RNA that flows through the nitrocellulose membrane.

Materials

Radiolabeled RNA pool (Support Protocol 1)
Binding buffer (see Critical Parameters)
Target protein

65° to 75°C thermal cycler, water bath, or heat block
Minifold I Dot-Blot System (Whatman)
Nylon transfer membrane (Hybond N+, GE Healthcare Life Sciences)
0.45-μm nitrocellulose transfer and immobilization membrane (BA85 Protran, Whatman)
Clean forceps or tweezers
PhosphorImager (GE Healthcare Life Sciences) and screen *or* X-ray film and densitometer (also see *APPENDIX 3A*)
Graphing software (e.g., SigmaPlot, Systat Software, or R Project)

Additional reagents and equipment for phosphor imaging or imaging using X-ray film and densitometry (*APPENDIX 3A*)

NOTE: All solutions and buffers should be made with deionized water (18.2 MΩ resistivity), filtered through 0.2 μm polyethersulfone (PES) membrane, and sterilized by autoclaving. Use sterile, disposable plasticware and micropipet tips with filter barriers where possible. See Chapter 4 introduction and *UNIT 4.1* for guidelines on standard methods to protect against contaminating RNases. If ribonuclease contamination is found to occur, it may be eliminated by treating water with diethylpyrocarbonate (DEPC; see *UNIT 4.1*).

Set up binding reactions

1. Collect the RNA precipitate by centrifugation and resuspend the radiolabeled RNA in a minimal volume (i.e., 5 to 10 μl) of RNase-free water. Dilute the RNA sample with binding buffer to a final concentration of 100 pM.

   The binding assay will yield 11 data points in triplicate (see below). Since each data point will be generated from a 50-μl binding reaction, 2 μl of the RNA solution should be adequate. If the specific activity of the RNA is not high enough, a higher concentration of RNA may be used; however, that will complicate the assumption that RNA is limiting and hence make the calculation of the K_d more difficult.

2. To ensure that each species in the RNA pool folds into the most accessible or most stable conformation, heat the RNA pool in 25 μl binding buffer to 65° to 75°C for 3 min and then allow the sample to cool to room temperature over ~10 min.

3. Add 25 µl of the protein target in binding buffer to the thermally equilibrated RNA from step 2. Use ten different protein concentrations in triplicate, ranging from 1 µM to 50 pM. Also include one data point with no protein to measure the filter-binding ability of the pool itself.

   The original protein solution should be sufficiently concentrated for all of the dilutions. To ensure consistency between samples, serial dilutions of the 1 µM sample can be made. The authors suggest the following final concentrations (i.e., 1 µM, and subsequent 1/3 dilutions): 1 µM, 333 nM, 111 nM, 37 nM, 12 nM, 4.1 nM, 1.4 nM, 460 pM, 152 pM, 51 pM, and a "no-protein" control. For statistically significant results, perform the binding assay in triplicate.

4. Incubate the binding reaction at room temperature for 15 min to 1 hr (see Critical Parameters).

Perform filter binding

5. Assemble the Minifold 1 Dot-Blot apparatus (Fig. 24.3.2). Lay the nylon transfer membrane on top of the perforations in the middle section. Moisten the nylon membrane and lay the nitrocellulose membrane on top of the nylon membrane, taking care to avoid the formation of bubbles between the two membranes. Cover and tighten the brackets.

   Prior to filtering the binding reactions, prewash the wells with binding buffer and check for leaks. When the manifold is used in conjunction with a water aspirator, turn the water faucet to a level that causes liquid to pass slowly through the membranes (i.e., 100 µl every 3 sec).

   Since there are so many binding reactions, it is more convenient to use a manifold apparatus that can accommodate multiple filtrations (up to 96 slots) than to assemble 33 individual filter holders.

6. Filter the binding reactions and wash three times, each time with 1 volume of binding buffer.

   When pipetting onto the manifold, dispense the liquid slowly and evenly. Try to keep the membrane constantly hydrated during each wash step. Keep the micropipet tip close to the membrane to avoid bubble formation, but not so close as to risk damaging the membrane.

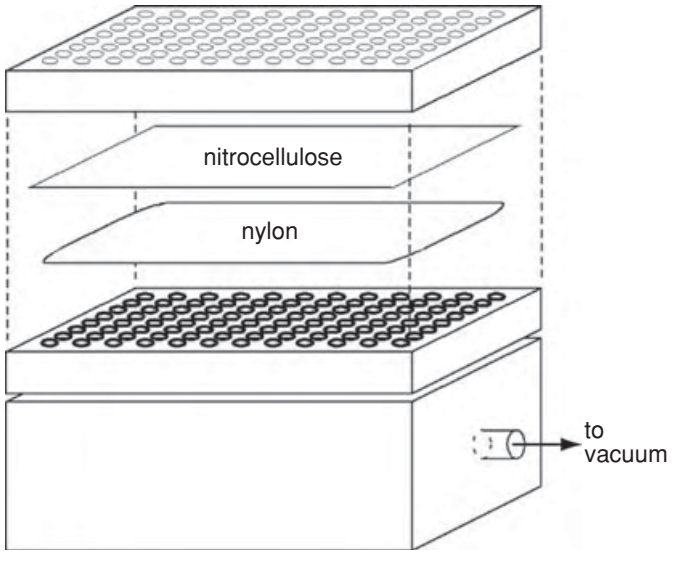

Figure 24.3.2 Assembly of the Minifold 1 Dot-Blot Milliblot apparatus used for binding assays. The nitrocellulose sheet collects binding species, whereas the nylon collects all remaining RNA. The apparatus is assembled, clamped down to hold the filters in place, then attached to a vacuum for filtration.

Utilization of a multichannel micropipettor (Pipet-Lite with LTS, Rainin) for the prewash and wash steps is recommended. Alternatively, the entire wash volume can be added to the blot at once.

7. Disassemble the manifold apparatus and transfer the membranes to a clean paper towel. Dry for ~5 min at room temperature or in an 80°C oven. Handle membranes with a clean pair of forceps or tweezers.

8. Cover membranes with plastic wrap and expose to a phosphor screen (e.g., PhosphorImager) or X-ray film for 4 to 12 hr (also see *APPENDIX 3A*).

   If the samples have a very high specific activity, the exposure time can be reduced to between 5 and 60 min.

9. Measure the radioactivity using the PhosphorImager, or a densitometer if X-ray film was used to develop the image, and calculate the binding percentages as follows:

   Fraction bound = cpm on nitrocellulose/(cpm on nitrocellulose + cpm on nylon)

   If X-ray film was used to develop the image, then a digitizer (densitometer) should yield similar results to those obtained with a PhosphorImager.

10. Plot the fraction bound as a function of the concentration of unbound protein. Fit the points to a curve using graphing software (e.g., SigmaPlot) and obtain a value for the aggregate parent dissociation constant. Within the SigmaPlot program, fit the curve using the equation $y = m_1 m_0/(m_0 + m_2)$, where y = the fraction of RNA bound, m_0 = concentration of unbound protein, m_1 = the extrapolated activity of the RNA at an infinite protein concentration (maximal value of fraction bound), and m_2 = the apparent dissociation constant.

    The apparent K_d is equal to the concentration of unbound protein at half the maximal value of fraction bound.

BASIC PROTOCOL 2

ISOLATING A FUNCTIONALLY ENRICHED POOL OF RNA

In the following protocol, the RNA pool is partitioned to isolate those species that bind to the target protein and not to the filter. RNAs that are coimmobilized with the target are eluted from the filter under denaturing conditions and subsequently isolated and amplified.

Materials

RNA pool (see Basic Protocol 1)
Binding buffer (see Critical Parameters)
Elution buffer (see recipe)
3 M sodium acetate (*APPENDIX 2*)
70% and 95% ethanol
25:24:1 phenol/chloroform/isoamyl alcohol saturated with 10 mM Tris·Cl, pH 8.0/1 mM EDTA (*UNIT 2.1A*), ice-cold (optional)
Chloroform (optional)
Isopropanol (optional)
65° to 75°C, 95°C. and 100°C heat blocks with appropriate bore sizes for the microcentrifuge tube
13 mm Nuclepore Pop-Top or Swin-Lok Filter holders (Whatman)
13-mm, 0.45-μm HAWP nitrocellulose disk filters (Millipore)
5-ml syringe
Vacuum manifold
Sterile forceps

NOTE: All solutions and buffers should be made with deionized water (18.2 MΩ resistivity), filtered through 0.2 μm polyethersulfone (PES) membrane, and sterilized by autoclaving. Use sterile, disposable plasticware and micropipet tips with filter barriers where possible. See Chapter 4 introduction and *UNIT 4.1* for guidelines on standard methods to protect against contaminating RNases. If ribonuclease contamination is found to occur, it may be eliminated by treating water with diethylpyrocarbonate (DEPC; see *UNIT 4.1*).

Partition the pool

1. Use >400 pmol of the RNA pool (>2.4×10^{14} different sequences) for selection.

   Using significantly lower quantities of RNA may affect the diversity of the population in the initial rounds of selection. Using significantly higher quantities may lead to precipitation of the nucleic acid pool. Irvine et al. (1991) devised a formula to determine the optimum protein and RNA concentration in order to minimize the number of rounds of selection, based on the K_d of the starting pool, the desired K_d, and the fraction of free RNA molecules that partitions as nonspecific background versus the fraction of RNA molecules that forms specific RNA:protein complexes. Empirically, the concentrations of many available protein targets will be in the nanomolar range, and a 1- to 10-fold excess of the RNA pool should suffice for early rounds of selection.

   If only a small amount of RNA pool is initially recovered from the gel, be sure to save at least some sample for the "no-protein" control (see below).

2. To ensure that each species in the RNA pool folds into the most accessible or most stable conformation, heat the RNA pool in 50 to 100 μl binding buffer (see Critical Parameters for discussion on choosing a binding buffer) between 65° and 75°C for 3 min, and then allow the sample to cool to room temperature over ~10 min.

   Since ionic strength, monovalent and divalent cation concentrations, pH, temperature, and buffer concentrations can all influence interactions with the target, it is usually wise to keep all of these parameters constant during the early rounds of selection when productive binding species are accumulating. Hence, the binding buffer, equilibration time, and preparation of the RNA for selection should be kept uniform until a significant interaction between pool and target is observed (see Critical Parameters for discussion of stringency of selection).

   Higher temperatures can be used for thermal equilibration, but the presence of divalent metal ions in the selection buffer can lead to RNA degradation.

3. Prior to the addition of the protein target, perform a negative selection to remove any filter-binding species that may be in the population. Place a pre-wetted filter into the filter holder top and lock the filter holder base into the clips protruding from the filter holder top. Secure the filter holder base by passing the ring lock down the filter holder top until it fits snugly (Fig. 24.3.3).

   Negative selection to remove filter-binding species is an extremely important step in the selection procedure. Filter-binding species are typically more numerous in a naive RNA population than are aptamers. If filter-binding species are not efficiently sieved from the population, they will quickly accumulate to the point where it may be difficult (and likely impossible) to select protein-binding species. If the potential for accumulating filter-binding species is large (i.e., the target has a low initial affinity for a pool, or selections with DNA or modified RNA pools), then repeat the preselection filtration to remove any filter-binding species that may persist, or carry out a post-selection filtration (see optional steps 17 through 20, below). If filter-binding species accumulate during a selection experiment, it is usually best to repeat the selection starting with a different pool that can be amplified with different primers.

   In addition to filter-binding species, replication parasites (see Critical Parameters for discussion on parasites) can accumulate in and over-run a selected population. A separate regime is required to avoid these selection predators.

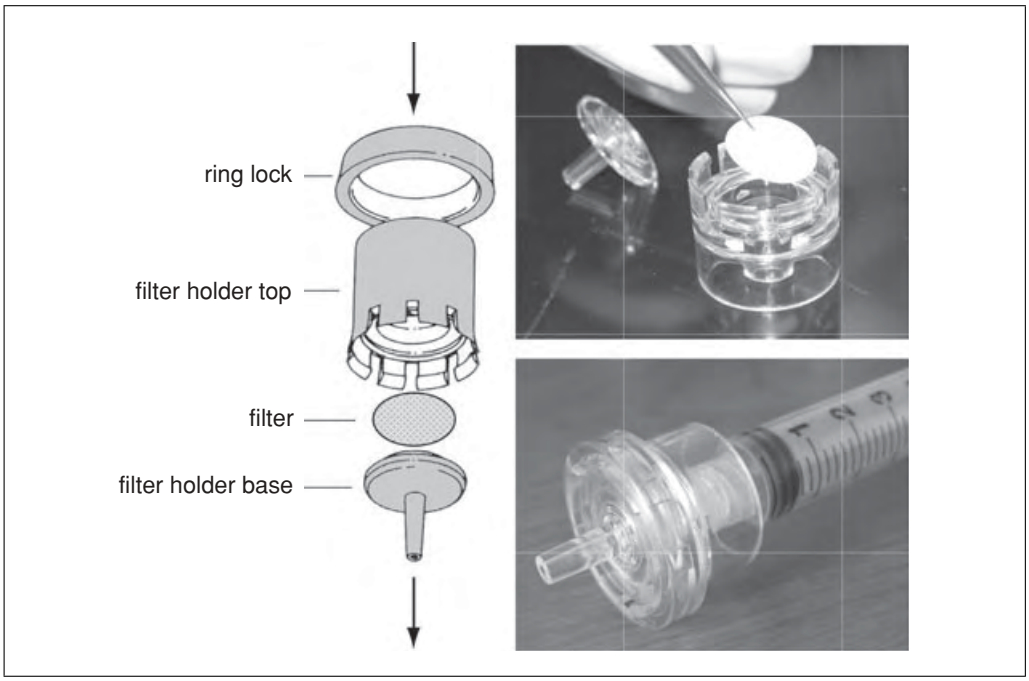

Figure 24.3.3 Components and assembly of filter holder used during selection. Pop-top graphic adapted, with permission, from Whatman product sheet.

4. Load the binding buffer onto the filter. Place the micropipet tip just above the filter to avoid the formation of any bubbles. Lock a 5-ml syringe to the top of the filter holder and apply gentle pressure to force the liquid out of the filter holder and into a collecting tube.

   If the syringe plunger does not regain position when pressure is removed, there is likely a leak in the filter. It should be removed and replaced with another filter.

   Prior to filtering the RNA, it is important to wash the nitrocellulose filter disk with binding buffer and check for leaks in the assembled filter holder. The syringe should form a tight seal with the filter holder. The pressure applied should be just enough to force the liquid through without rupturing the membrane. Formation of foam at the bottom of the filter holder or the presence of a hissing sound when pressure is applied indicates that the pressure is too high, and the integrity of the seal or the membrane may have been breached. Test for leaks every time the filter holder is assembled to avoid substantial loss of sample.

5. Load the RNA solution onto the filter. Place the micropipet tip just above the filter to avoid the formation of any bubbles. Lock a 5-ml syringe to the top of the filter holder and apply gentle pressure to force the liquid out of the filter holder and into a collecting tube.

   Since there will still be some amount of liquid retained by the filter and filter holder, it is necessary to wash the filter with an equal amount of binding buffer to maximize the collection of non-filter-binding species. Discard the filter.

6. Add the protein target and any competitors, specific and/or nonspecific, to the filtrate. Allow the binding reaction to equilibrate (typically 30 min initially; however, this time can be reduced when selecting for enhanced binding kinetics).

   In selection experiments that targeted the cytokine bFGF, the authors used an equimolar protein-to-RNA ratio for the first two rounds of selection and decreased it 10-fold after two rounds and 60-fold after another two, yielding a functionally enriched pool after six rounds of selection and amplification (Table 24.3.1). The final volume of the binding reaction should be from 100 to 200 μl. In addition, to ensure that the selected RNAs

Table 24.3.1 Progress of N30 Selection Against bFGF[a,b]

Round	Input RNA (nM)	Input bFGF (nM)	RNA:bFGF	% bound to protein	% bound to filter
1	800	760	1.05	2.1	2.3
2	800	760	1.05	—	—
3	800	76	10.5	—	—
4	800	76	10.5	6.0	4.0
5	800	13	61.5	—	—
6	800	13	61.5	17.0	0.4

[a]Pools were assayed in a 50-µl reaction at a concentration of 75 nM in the presence and absence of equimolar protein.
[b]N30 is a RNA pool with 30 random sequence positions (Lato et al., 1995).

are actually binding to the target and not to the filter, a parallel binding reaction in the absence of protein can be carried out intermittently. The authors strongly suggest that "no-protein" controls be scrutinized before the selection begins, and then after every three additional rounds of selection (i.e., rounds 3, 6, and 9).

The choice of selection conditions is probably the second most important factor (following the choice of target) for determining the success of a selection experiment. While general guidelines for modulating the stringency of selection can be recommended (see Critical Parameters for comments on the stringency of selection), every target and every selection is different and no precise guidelines for success can be provided. In general, the stringency of selection should be lower in early rounds of selection and higher in later rounds. This will give binding species an opportunity to establish themselves in the population relative to filter-binding species.

It should be noted that there is some danger of cross-contaminating the selected pool with the "no-protein" control. Basically, executing the "no-protein" control is identical to selecting for protein-independent (filter) binding species; hence, DNA arising from the "no-protein" control should be handled with care.

7. During the equilibration, assemble a second filter disk into a holder (see step 3).

8. Load the equilibrated binding reaction onto the filter. Place the micropipet tip just above the filter to avoid the formation of any bubbles. Lock a 5-ml syringe to the top of the filter holder and apply gentle pressure to force the liquid out of the filter holder and into a collecting tube.

   If the syringe plunger does not regain position when pressure is removed, there is likely a leak in the filter. It should be removed and replaced with another filter. The solution in the collection tube can be reapplied to the new filter.

9. Wash the unbound or weakly bound pool.

   Three washes are sufficient during early rounds.

   Alternatively, the filter holder can be attached to a vacuum manifold (which is used here to maintain a constant negative pressure during filtration, so that each round of selection is similar and reproducible). Apply a negative pressure of 127 mm of Hg to the filter holder. Pipet the binding reaction directly onto the filter with the tip just above the filter, avoiding the formation of bubbles, which may lead to an uneven application of the sample to the filter and impede the flow of liquid through the filter. Wash the filter with 3 vol of binding buffer.

   Varying the strength of the vacuum, uneven application of the sample, and formation of bubbles during wash steps may result in inefficient sieving of binding from nonbinding species, and hence may reduce the efficiency of an individual round of selection. However, the selection as a whole is fairly robust with respect to changes in these parameters. In other words, even if steps are not performed perfectly, the selection can be carried forward.

It should be noted that the vacuum manifold attachment must be thoroughly cleaned after each round or target. Nonbinding RNAs can stick to the manifold and transfer to the filter holder base in alternating selection experiments, thereby contaminating them during elution. The authors recommend using a green Scotch-Bright pad (3M Company) to scrub the manifold with Alconox Precision Cleaner and water. The manifold should then be rinsed with water and dried by spraying with ethanol.

Elute RNA off the filter

10. Remove the filter containing RNA:protein complexes from the filter holder using sterile forceps and place it in a 0.5-ml microcentrifuge tube. Transfer the filter quickly to avoid ribonuclease contamination from the surrounding environment.

    The authors strongly recommend changing gloves after this step to prevent the accumulation of contaminating RNAs in solutions and on equipment.

11. Add 200 µl of elution buffer and heat for 5 min at 95°C, followed by agitation (vortexing) to elute RNA molecules from the protein and filter. Transfer the eluate to a separate tube and repeat elution with fresh elution buffer.

    Two shorter, smaller-volume elutions will more efficiently recover intact RNA than one long, large-volume elution.

12. Ethanol precipitate the RNA by adding one-tenth volume of 3 M sodium acetate (0.3 M final), 3 µl of 1 mg/ml blue-dyed glycogen, and 2.5 volumes of 95% ethanol, microcentrifuging, and washing the pellet with 70% ethanol (see Basic Protocol 1, steps 9 and 10).

    If the binding buffer contains a high (>0.5 M) salt concentration, dilute the eluate with an equal volume of RNase-free water and precipitate with one volume of isopropanol instead.

    If a subsequent phenol/chloroform extraction is necessary, this precipitation can be omitted.

Perform a phenol/chloroform extraction (optional steps)

13. To remove residual peptide fragments or proteins that may have coeluted with the RNA, add an equal volume (i.e., 400 µl) of cold 25:24:1 phenol/chloroform/isoamyl alcohol. Vortex, then microcentrifuge for 1 min at maximum speed to separate the liquid phases (the RNA should be in the top, aqueous phase). Transfer the aqueous phase to a new 1.5-ml microcentrifuge tube.

    Avoid transferring phenol/chloroform with the aqueous layer, as it can interfere with subsequent enzyme reactions. Nevertheless, the aqueous phase will sometimes appear milky, especially at low temperatures, due to the presence of dissolved phenol-chloroform.

14. Extract the eluate with a similar volume of chloroform to remove any residual phenol.

    Avoid transferring chloroform with the aqueous layer, as it can interfere with subsequent enzyme reactions.

15. Dilute the eluate with an equal volume (~400 µl) of RNase-free water and add 800 µl of isopropanol, then chill 20 min at −20°C to precipitate.

    A carrier such as glycogen (see step 12) can be added to aid precipitation.

    The elution buffer contains a high concentration of urea. Dilution with 400 µl water and precipitation with isopropanol is necessary to avoid the formation of salt precipitates, which appear as oily, unstable droplets in the bottom of the microcentrifuge tube following centrifugation. If such "salt pellets" appear, additional water should be added to the sample, the mixture should be homogenized, and the precipitation repeated.

16. Microcentrifuge 30 min at maximum speed, remove the supernatant, and resuspend the RNA sample in 12 ml sterile RNase-free water.

Perform an additional negative selection (optional steps)

An extremely effective method for ridding the population of filter-binding species is to carry out an additional negative selection following the selection for binding species, but prior to amplification. However, at early stages of the selection, an additional post-selection filtration step may reduce the complexity of the selected population. Therefore, it is recommended that post-selection filtration only be carried out following the second round of selection. Post-selection filtration can also be used to successfully remove filter-binding species that have begun to accumulate and overrun a selected population. However, once filter-binding species have established themselves, even a combination of pre- and post-selection filtrations may not allow specific binding species to regain a selective advantage. If a simple regime of pre- and post-filtration negative selections does not succeed in drastically reducing or eliminating established filter-binding species, the selection should be repeated with a different RNA pool that can be amplified with different primers, as recommended above.

17. Resuspend the selected RNA pellet in 50 μl binding buffer.
18. Assemble the filter holder with a fresh filter disk as described above.
19. Filter the sample and wash as described above.
20. Discard the filter disk and ethanol precipitate the RNA filtrate as described in step 12.

    A carrier (glycogen; see step 12) can be added to improve the efficiency of precipitation.

    If the binding buffer contains a high (>0.5 M) salt concentration, dilute the filtrate with an equal volume of RNase-free water and precipitate with isopropanol instead (see step 15).

AMPLIFYING SELECTED PROTEIN-BINDING RNA SPECIES

BASIC PROTOCOL 3

In the following steps, RNA species that survived the positive and negative selection steps are reverse transcribed to generate a cDNA library, which is subsequently amplified by PCR. The double-stranded DNA resulting from these steps comprises the pool from which the next round of selection will begin. While the authors have found that reverse transcription and PCR steps can be combined for some selections, this is not universally true. To obtain the highest yield of RNA and DNA products, it is frequently desirable to carry out separate reverse transcription and PCR reactions, as described below.

Materials

Selected RNA pool (Basic Protocol 2)
TE buffer, pH 8.0 (see recipe), or RNase-free water
SuperScript II reverse transcription kit (Invitrogen)
20 and 200 μM 3′-end primer
4 mM dNTP mix (containing 4 mM each of dATP, dCTP, dGTP, and dTTP)
10× PCR buffer (see recipe)
20 μM 5′-end primer
5 U/μl *Taq* DNA polymerase (New England Biolabs)
6× nondenaturing dye: 0.6% (w/v) bromphenol blue and 10× ethidium bromide in TBE buffer (see *APPENDIX 2* for TBE buffer)
NuSieve agarose (Cambrex)
10 mg/ml ethidium bromide solution (*APPENDIX 2*)
TBE buffer (*APPENDIX 2*)
3 M sodium acetate (*APPENDIX 2*)
1 mg/ml blue-dyed glycogen (GlycoBlue; Ambion)

70% and 95% ethanol
Thermal cycler (e.g., BioRad DNAEngine with heated lid) and PCR tubes

Additional reagents and equipment for the polymerase chain reaction (Chapter 15), agarose gel electrophoresis (e.g., *UNIT 2.6*), and DNA sequencing (Chapter 7)

NOTE: All solutions and buffers should be made with deionized water (18.2 MΩ resistivity), filtered through 0.2-μm polyethersulfone (PES) membrane, and sterilized by autoclaving. Use sterile, disposable plasticware and micropipet tips with filter barriers where possible. See Chapter 4 introduction and *UNIT 4.1* for guidelines on standard methods to protect against contaminating RNases. If ribonuclease contamination is found to occur, it may be eliminated by treating water with diethylpyrocarbonate (DEPC; see *UNIT 4.1*).

Reverse transcribe the selected binding species into ssDNA

The reverse transcription (RT) and PCR amplification should be performed in separate steps so that the accumulation of DNA during a cycle course can be evaluated.

1. Resuspend the RNA in 13 μl TE buffer or RNase-free water and set up the following 20-μl RT reactions:

   8.5 μl RNA suspension
   2.0 μl 200 μM 3′-end primer
   2.5 μl 4 mM dNTP mix

   Perform the following controls in parallel with the amplification of selected RNA species in order to detect nonspecifically bound RNA species and replication parasites (see Critical Parameters for discussion of parasites).

   a. *No-template control:* To ensure that none of the stock solutions have been contaminated with exogenous RNA or DNA amplicons, set up an RT-PCR reaction without adding any template.
   b. *No-RT control:* To ensure that amplified products are in fact derived from selected RNA species and not from endogenous or cross-contaminating DNA molecules, set up an RT-PCR reaction without the reverse transcriptase.

2. Heat denature the reaction at 65°C in a thermal cycler for 5 min and cool to room temperature over 10 min

   This step ensures that primer can access and anneal to the primer-binding site on the pool.

3. Add the following components to each reaction:

   4 μl 5× First Strand Buffer (from SuperScript II kit)
   2 μl 0.1 M DTT (from SuperScript II kit)
   1 μl SuperScript II reverse transcriptase from SuperScript II kit (or RNase-free water for the no-reverse transcriptase control)

4. Mix the reaction well by pipetting up and down, then incubate the reaction at 42°C for 50 min. Heat inactivate the enzyme at 70°C for 15 min.

Perform cycle-course PCR

It is important not to over-amplify the selected templates, especially in the first several rounds, since amplification artifacts can dominate a selection. To determine the optimal number of cycles for amplification in each round, an initial "ranging" or cycle-course PCR must be performed. A small sample of DNA is taken from the PCR every 2 to 3 cycles, and saved for gel analysis. The cycle at which a strong band is present is the

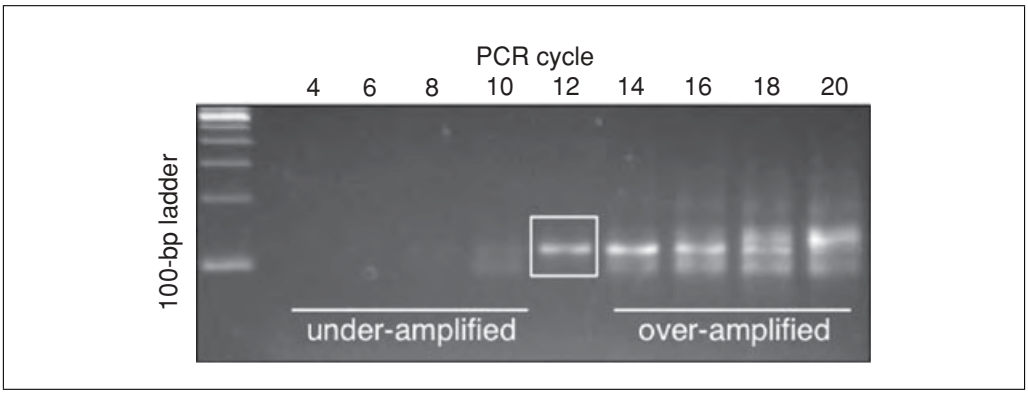

Figure 24.3.4 Cycle-course PCR.

cycle that should be used to amplify the remainder of the pool (see Fig. 24.3.4). If a "no-protein" negative control selection was performed, the relative appearance of bands during the cycle course can be used to help determine if partitioning of binding species from nonbinders has occurred.

5. With the ssDNA from the reverse transcriptase reaction, set up the PCR reaction as follows:

   10 µl 10× PCR buffer
   5 µl 4 mM dNTP mix
   2 µl 20 µM 5′-end primer
   2 µl 20 µM 3′-end primer
   2 µl pool ssDNA (from step 4)
   0.5 µl 5 U/µl *Taq* DNA polymerase
   77.5 µl nuclease-free water.

6. Incubate the reaction under the following conditions:

1 cycle:	5 min	95°C	(initial denaturation)
20 cycles:	45 sec	92°C	(denaturation)
	45 sec	50°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	indefinitely	4°C	(hold).

   It should be noted that the listed conditions have been optimized for the pool design methods described in UNIT 24.2. However, different pools and primers may require very different amplification conditions. See UNIT 15.4 for comments on primer selection and for the experimental parameters that govern reverse transcription and PCR.

7. Within the last 10 sec of the 72°C extension step in cycle 6, remove a 5-µl sample and combine it with 1 µl of 6× non-denaturing dye in a separate PCR tube.

   To prevent cross-contamination within the thermal cycler, remove the tube when aliquotting each sample.

8. Repeat step 7 at the end of the 72°C extension step of cycles 8, 10, 12, and 14. Allow the PCR to progress to cycle 20 and remove a final 5-µl sample aliquot within the last 10 sec of the 72°C extension step of that cycle.

Check for the presence of amplified, double-stranded DNA

9. Make a 3.8% NuSieve agarose gel solution that contains 0.1 µg/ml ethidium bromide. Pour an agarose gel with this solution. Load the samples and run the gel in TBE at 125 V for 30 min (see *UNIT 2.6*). Look for products with a UV transilluminator (see Fig. 24.3.4).

An estimate of the minimal number of cycles needed to visualize a product band on the agarose gel can be roughly calculated. Consider that, of the 5 μg of RNA added to the selection, ~3% likely binds to the filter and is lost during the negative selection step. Approximately 0.1% to 1% of the population may bind to the target. When the selected RNA is precipitated, two-thirds of the sample are used for the reverse transcription and one-tenth for the PCR. Therefore:

(5.0 μg)(0.97)(0.01)(2/3)(0.1) = 3.2 ng RNA.

Assuming that every cycle doubles the amount of DNA, a minimum of nine cycles would be necessary to obtain 1 to 2 μg of DNA. This would imply that 0.05 to 0.1 μg could be loaded and readily visualized on the ethidium bromide–stained agarose gel. Thus, from 10 to 12 cycles should initially be carried out and the products analyzed by gel electrophoresis. The authors frequently find this rough estimate to be accurate.

The accumulation of double-stranded DNA is closely monitored in order to avoid over-amplification of the sample and the concomitant accumulation of high-molecular-weight species. DNA that has been over-amplified will look blurry and dispersed following analysis by gel electrophoresis. These large DNA molecules are often the result of the 3′ end of a single-stranded DNA folding back and internally priming its own extension, resulting in a long stem-loop that can be amplified by a single PCR primer (also known as single-primer artifacts). Over-amplified DNA templates can also yield RNA molecules of the incorrect size following transcription.

If one primer is more abundant or efficient than the other, a smaller, single-stranded DNA band or bands may also be present.

The various controls (no-protein, no-template, no–reverse transcriptase) should be amplified in parallel with the actual sample. If specifically bound RNA is acting as a template for the accumulating amplicons, then the "no-RT" sample should lag the pool PCR reaction by at least three cycles. It is desirable that no bands be observed in the "no-template" control, but if they do arise, they should lag the RT-PCR reaction by at least five cycles. If bands do arise, a distinction should be made between full-length PCR products (indicating contaminating replicons) and smaller products (likely primer amplification artifacts). If product bands in the control lanes are as prominent as product bands in the experimental lanes, then it is necessary to check or remake reagents and go back and repeat the previous round of selection. There is one exception to this rule: in the initial rounds, it is common to see a band in the "no-protein" control lane because the proportion of the population that binds to the filter is typically greater than the proportion that binds specifically to the target. However, subsequent rounds of selection should result in the diminution or disappearance of the "no-protein" band.

Observing the number of cycles needed to visualize a double-stranded DNA band can loosely monitor the progress of the selection. The number of cycles should be roughly proportional to the amount of RNA pool that originally binds to the protein. Therefore, if the RNA eluted from the "no-protein" control requires more cycles for full amplification than does the RNA selected in the presence of protein, it can be tentatively assumed that the selected RNA is binding to the protein. Occasionally, in the early rounds of selection, this may not be true, since a very small fraction of the pool will bind to the protein relative to the small fraction of the pool that adheres to the filter.

Counting PCR cycles is, however, only a very rough (and frequently inconsistent) measure of success. In fact, it is common for the number of cycles required to fully amplify selected nucleic acids to vary greatly between rounds. Direct binding assays of the RNA pool (Support Protocol 3) are a much more accurate and useful gauge of the progress of a selection experiment.

10. Once the optimum PCR cycle has been determined, set up eight 100-μl PCR reactions as described below, and perform the cycling conditions listed above in parallel for the optimum number of cycles.

    80 μl 10× PCR buffer
    40 μl 4 mM dNTP mix
    16 μl 20 μM 5′-end primer

16 μl 20 μM 3′-end primer
16 μl pool ssDNA
4 μl 5 U/μl *Taq* DNA polymerase
628 μl nuclease-free water

11. Ethanol precipitate the RNA by adding one-tenth volume of 3 M sodium acetate (0.3 M final), 3 μl of 1 mg/ml blue-dyed glycogen, and 2.5 volumes of 95% ethanol, microcentrifuging, and washing the pellet with 70% ethanol (see Basic Protocol 1, steps 9 and 10).

Use amplified DNA template for the next round of selection

12. Resuspend the pellet in 20 μl TE buffer or nuclease-free deionized water. Proceed with the next round of selection starting with step 1 of Basic Protocol 1.

A 100-μl PCR reaction yields ~1 μg dsDNA, so approximately one-quarter of the resuspended DNA will equate to 2 μg sample and should be used for the next transcription reaction. The remaining dsDNA, and potentially the remaining RNA after transcription, can serve as a long-term, archival sample.

ASSAYING THE ACCUMULATION OF PROTEIN-BINDING RNA SPECIES

SUPPORT PROTOCOL 3

To verify that the RNA pool has been or is being winnowed to those few sequences that bind the protein target with high affinity and specificity, the selected RNA pool should periodically be assayed for its ability to bind the target protein. The authors recommend an initial binding assay after five rounds of selection and amplification, then again every three additional rounds (the same recommendation that was made with regard to checking for filter-binding species; the two tests can be carried out in parallel). While the initial binding assay is carried out at a series of protein concentrations to gauge the amount of protein that should be used in the selection, the progress of the selection can be most simply monitored by internally radiolabeling the RNA and determining how much binds to a single, convenient concentration of the protein target.

Materials

Pool of dsDNA after *n* rounds of selection (Basic Protocol 3)
Binding buffer (see Critical Parameters)
Target protein
167 mCi/ml [α-^{32}P]ATP (7000 Ci/mmol; ICN Biomedical Inc or GE Healthcare Life Sciences)

Additional reagents and equipment for purifying a radiolabeled RNA pool (see Basic Protocol 1) and performing the filter binding assay (see Support Protocol 2)

1. Generate radiolabeled RNA pool via a "hot transcription" with α-labeled nucleoside triphosphate (typically [α-^{32}P]GTP or ATP) and purify as described previously (Basic Protocol 1).

The transcription is carried out as described in Basic Protocol 1 except that 1 μl of α-labeled ATP is added to the 20-μl transcription reaction in addition to the standard NTP mix.

After the RNA has been separated from free nucleotides via PAGE, the buffer in the bottom chamber will contain unincorporated nucleoside triphosphates and will therefore be extremely radioactive. Care should be taken when transferring and disposing of this solution.

2. Thermally equilibrate 1 μg of the radiolabeled RNA pool after a round of selection in binding buffer as described in Support Protocol 2, steps 1 and 2.

3. For each round tested, set up reactions in triplicate with and without target. Add an equimolar amount of protein to the RNA pool. Incubate the binding reaction under conditions similar to those used for selection.

   The binding reaction volume should be the same as that used for the selection. If the amount of protein sample is limited or limiting, less protein can be used in the binding reaction. However, one should be cognizant of the fact that less than 100% binding is possible. Alternatively, less protein and less RNA sample can be used, although the diminution of both components will mean that one is assaying binding under conditions more stringent than those actually used for selection. While the volume of the binding reaction could also be diminished to conserve protein, it is difficult to uniformly apply volumes less than 30 μl to the filter.

   To limit spurious background signal, blocking agents such as nonradioactive tRNA and BSA can be added to the binding reaction, or added immediately prior to filtration.

4. Filter each binding reaction and wash three times, each time with 1 volume binding buffer (see Support Protocol 2, steps 5 through 10).

   A good result at this point would be 15 to 20% fraction bound above background (see Table 24.3.1. round 6). If binding to filter alone is too high, then filter binders are being selected and more negative selection is needed.

5. If the desired binding is detected, clone (*UNIT 15.4*) and sequence the pool (see Chapter 7) to isolate individual variants.

6. Compare aptamers with one another to identify sequence and structural similarities.

   A typical observation is the selection of sequence families that are similar over a large portion of the aptamer and/or short sequence motifs that are common to multiple, otherwise different aptamers.

REAGENTS AND SOLUTIONS

Use RNase-free deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Denaturing dye, 2×

TBE buffer (*APPENDIX 2*) containing:
0.1% (w/v) bromphenol blue
7 M urea
Store up to 6 months at −20°C

Denaturing polyacrylamide gel, 8%

TBE buffer (*APPENDIX 2*) containing:
8% (v/v) 19:1 acrylamide:bisacrylamide
7 M urea

See UNIT 2.12 for full details on pouring and running the gel.

Elution buffer

4 to 7 M urea
25 mM disodium EDTA
Store up to 3 months at −20°C

Prepare with RNase-free water.

PCR buffer, 10×

100 mM Tris·Cl, pH 8.4 (*APPENDIX 2*)
500 mM KCl
20 mM $MgCl_2$

PCR buffer can be stored at room temperature, or can be refrigerated or frozen. If it is frozen, care should be taken to mix the buffer after thawing.

TE buffer, pH 8.0

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
1 mM EDTA, pH 8.0 (*APPENDIX 2*)
Store up to 6 months at −20°C

COMMENTARY

Background Information

Sol Spiegelman and co-workers developed a working system for the in vitro replication and evolution of small RNA molecules over 35 years ago (Mills et al., 1967; Levisohn and Spiegelman, 1969; Kramer et al., 1974). The development of more advanced (although conceptually identical) methods for in vitro evolution, as described in this unit, was potentiated by advances in the chemical synthesis of oligonucleotides and the amplification of nucleic acids, such as PCR, in vitro transcription, and self-sustained sequence replication (3SR) (Guatelli et al., 1990). The adaptation of these methods to in vitro evolution of RNA molecules was partially due to the recognition that early evolutionary events, such as the genesis of ribozymes, could be recapitulated in a test tube, and partially due to the recognition that the ability to tailor RNA-binding species and catalysts might have numerous biotechnological applications. Following the publication of key papers outlining and proving selection technologies (Ellington and Szostak, 1990; Tuerk and Gold, 1990), a much wider array of selection experiments has been attempted. To date, RNA molecules that can bind targets as small as zinc and as large as viruses and organelles have been selected (reviewed in Stoltenburg et al., 2007 and Shamah et al., 2008). RNA molecules that interact with both nucleic-acid-binding proteins and non-nucleic-acid-binding proteins can be selected with almost equal facility from random sequence populations. These results have been thoroughly reviewed in numerous publications (Gold et al., 1995; Uphoff et al., 1996; Kulbachinskiy, 2007).

Critical Parameters

Choosing protein targets

As briefly described above, a wide variety of proteins have proven to be successful targets for selection experiments, including enzymes, transcription factors, cytokines, antibodies, and viral capsids (Gopinath, 2007; Stoltenburg et al., 2007). There is no common functional theme uniting these targets, nor can many generalities be drawn regarding their biochemistry or structure. However, it is safe to say that "good" selection targets tend to fall into two classes. First, proteins that normally bind nucleic acids will also be able to extract aptamers from a random sequence pool. The notion of a nucleic-acid-binding protein can, to some extent, be expanded to include proteins that bind nucleotides. For example, kinases and dehydrogenases bind nucleotide cofactors and have proven to be good selection targets.

Second, proteins that for whatever reason contain basic patches in their primary sequences or on their surfaces also frequently yield high-affinity aptamers. For example, many cytokines and other signal-transduction proteins bind heparin or other sulfated oligosaccharides, and can also be used to select aptamers from random sequence populations. The anti-cytokine aptamers frequently bind to the same sites as heparin (Jellinek et al., 1993). Similarly, proteins that bind phosphate or phosphomonoester or phosphodiester bonds frequently have positively charged active sites and can be used to elicit aptamers. For example, anti-phosphatase aptamers have been selected from random sequence pools (Bell et al., 1998).

This is not to say that proteins that do not fall into these categories will necessarily be poor selection targets, but merely that they are not guaranteed selection targets. For example, antibodies have frequently proven to be excellent selection targets irrespective of whether they bind negatively charged antigens (Keene, 1996). This likely implies that proteins with large pockets or clefts on their

surface are good selection targets. This hypothesis is further bolstered by another line of reasoning. Aptamers selected to bind proteins frequently inhibit protein function. That is, anti-antibody aptamers block interactions with antigens, anti-enzyme aptamers inhibit enzymatic activities, and so forth. This so-called "homing principle" may be due to the fact that aptamers not only have to form a surface that is chemically complementary to a target, but they also must fold into a structure that properly presents the chemically complementary surface. The most informationally parsimonious way to achieve both functions is to fit into a pocket on a target, rather than to form a "grasping" structure that can enfold a surface protrusion of a target. Thus, the most common (and most highly represented) aptamers may be those that fit into surface crevices. In contrast, antibodies have a preformed structure for the presentation of chemically complementary surfaces, and thus can more easily grasp protruding epitopes and less easily fit into surface crevices.

Overall, researchers should be guided not so much by these considerations as by the results of initial binding assays with their particular protein target. If the target binds to the filter (not a given, since small, acidic proteins such as the Rop protein from *E. coli* will frequently pass through the filter) and shows some affinity for a random sequence pool, then it is highly probable that there will be some sequences or structures within the pool with greatly enhanced affinities for the target.

Choosing a binding buffer

The binding buffer should promote specific binding of nucleic acids to a protein target. The first consideration in choosing a buffer is to identify conditions under which the protein is active, or at least stable. In addition, if the selected nucleic acid species are to eventually be used in a particular environment, the selection buffer should reflect this environment. For example, if the selected nucleic acids are to be expressed in a cell, then the selection buffer should be at physiological pH and contain physiological ion concentrations. Second, there are a variety of parameters that can be used to make the RNA pool more or less "sticky." These parameters are discussed in much greater detail below (see Stringency of selection).

A typical binding reaction is built from one of the commonly used buffers, such as Tris·Cl, phosphate, or HEPES, which can hold the pH near 6 to 8, together with 50 to 200 mM NaCl or KCl and 1 to 10 mM $MgCl_2$. However, these are merely suggestions, and aptamers have in fact been selected under a variety of buffer conditions. For example, in the selection that targeted bFGF, phosphate-buffered saline was used even though it lacked divalent cations (Jhaveri, 1998). Similarly, ribozyme selections have been carried out in which a variety of divalent metal ions are mixed, and nascent ribozyme species "decide" which combination of metals most enhance their activities (Lehman and Joyce, 1993). An equivalent strategy could be used for the selection of aptamers.

Selection matrices

Due to the tremendous ratio of matrix surface area to protein surface area, matrix-binding aptamers can quickly and easily eclipse target-binding aptamers. Proteins are likely captured on nitrocellulose or modified cellulose filters via hydrophobic interactions. Nucleic acids are, by and large, too hydrophilic or charged to be similarly captured. This distinction is the basis for most filter-binding assays. However, the nucleobases of nucleic acids obviously contain large hydrophobic surface areas, and it is easy to select nucleic acids that can present nucleobases and be captured by the filter. Selected filter-binding sequences frequently contain purine (especially guanosine) tracts presented as single-stranded loops or bulges. Interestingly, hydrophobic-binding sequences selected on one hydrophobic matrix are frequently cross-reactive with other hydrophobic matrices: i.e., microtiter plate-binding species can bind tubes and filters, filter-binding species can bind tubes and microtiter plates, and so forth.

In order to avoid filter-binding sequences, the authors have filtered RNA samples multiple times in the absence of protein, and in some cases filtered samples following selection but prior to the RT-PCR step. Matrix-binding sequences can also be avoided by altering the matrices used for selection. For example, techniques such as gel mobility shifts, immunoprecipitation, and affinity chromatography have all been successfully used to sieve pools and select target-binding aptamers (Conrad et al., 1996). If filter-binding species predominate in a population even after appropriate precautions are taken, these alternative selection techniques can be used either to rid the selected population of the filter-binding species or, better yet, to restart the selection. For example, if the immunoprecipitation of RNA:protein complexes has been worked out in advance, then

immunoprecipitation can be interspersed with rounds of filter binding.

Even though the selection of filter-binding sequences can be a problem, filter binding is still generally recommended as the technique of choice for most selections. Gel mobility shift experiments tend to be much more sensitive to parameters such as sample preparation, ionic strength, pH, and electrophoresis conditions than are filter-binding experiments. Moreover, just as filter-binding species can be inadvertently selected during filtration selection, RNA species with altered electrophoretic mobilities (e.g., dimers) can be selected during gel-mobility shift selections. Immunoprecipitation experiments require an additional protein reagent, and consequently anti-antibody rather than anti-target aptamers are frequently selected. Affinity chromatography or similar techniques generally require that very large amounts of target proteins be committed to the preparation of affinity matrices. If affinity elution is to be used, then even larger amounts of target proteins will be required. Moreover, aptamers that bind to agarose matrices can be selected almost as easily as aptamers that bind to nitrocellulose or modified cellulose filters (although the two, thankfully, do not cross-bind to one another's matrices). Finally, microtiter plate panning selections encourage the accumulation of the same sorts of matrix-binding aptamers that are elicited by filter-binding selections.

Stringency of selection

Overall, most selection experiments are generally competitions between specifically and nonspecifically binding nucleic acid species. The authors tend to initially choose conservative binding conditions in hope of promoting the early establishment of binding species in the population. While this may mean that low-affinity species are isolated from the pool along with high-affinity species, the low-affinity species can eventually be removed by increasing the stringency of selection. In essence, time (the number of cycles required to purify high-affinity species) can be traded for the assurance that filter-binding species will not accumulate and predominate.

A variety of parameters can be modulated to increase or decrease the stringency of a selection experiment. These parameters should initially be chosen based on the results of Support Protocol 2, which assays the affinity of the pool for the target and should be made progressively more stringent based on the results of Support Protocol 3.

The amount of protein target. The more protein there is to bind, the easier it is to capture nucleic acid binding species. Using low amounts of protein increases competition among binding species. However, the amount of protein target available to researchers is usually limited, and thus it is easier to use a set amount of protein (usually from 0.1 to 10.0 μM per binding reaction) and to vary the RNA:protein ratio.

RNA:protein ratio. By increasing the ratio of pool to target, more binding species will compete for a smaller number of targets. Typically, after a few initial rounds with an equimolar pool-to-target ratio, the ratio is increased to between 10:1 and 100:1. This increase can be effected either by increasing the amount of RNA or by decreasing the amount of protein. Because of the underlying competition between specifically binding species and nonspecifically binding species, increasing the amount of RNA is preferable to decreasing the amount of protein. For a more detailed treatment of this subject, see Irvine et al. (1991). However, the general conclusions of these mathematical models are similar to the empirical advice given here.

Competitors. High concentrations of nonspecific, non-amplifiable competitors such as tRNA or bulk cellular RNA will compete with low-affinity binding species that adhere to basic patches on the surface of a protein. Typically, a 100-fold excess of tRNA is used. Similarly, specific competitors can be used to block the access of low-affinity binding species to a preferred site. Wild-type nucleic acid ligands can be used to block the binding sites of nucleic acid binding proteins. For example, during the selection of anti-Rev aptamers, Giver et al. (1993) included a 10-fold excess of the wild-type Rev-binding element. The anti-Rev aptamers that were obtained could bind with high affinity to the RNA-binding domain of Rev and could effectively compete with the wild-type Rev-binding element. Other ligands or substrates can also be used to block the binding or catalytic sites of non-nucleic acid-binding proteins. For example, during the selection of anti-bFGF aptamers, Jellinek et al. (1993) included heparin, a natural ligand for bFGF. The anti-bFGF aptamers that were obtained could bind with high affinity to the heparin binding site and could effectively compete with heparin.

Cation concentration. Monovalent cations (such as Na^+) and divalent cations such as Mg^{2+} stabilize the structure of RNA molecules and contribute to both specific

and nonspecific binding. Decreasing monovalent and/or divalent cation concentrations, therefore, can increase the stringency of the selection. However, it is unclear, in advance, whether specific or nonspecific binding species will be more favored by such a change. Moreover, since binding species that require a monovalent and/or divalent cation to fold into shapes that are chemically complementary to a target may be favored in the early rounds of selection, potentially high-affinity binding species may be lost by changing the binding buffer late in the selection experiment. It is better to attempt to change the buffer dependency of aptamers by partial randomization and reselection following the initial selection experiment, rather than to attempt to change the buffer dependency during the selection.

Conversely, higher concentrations of monovalent cations (generally Na^+ or K^+) increase the structural integrity of folded nucleic acids by neutralizing the close approach of nucleic acid strands. However, higher monovalent ion concentrations also suppress electrostatic interactions with targets. Thus, paradoxically, both "low" and "high" monovalent ion concentrations can be used to increase the stringency of a selection experiments. Higher concentrations of divalent cations such as magnesium help to maintain the structural integrity of RNA molecules and potentially facilitate the formation of salt bridges between acidic residues and the phosphate backbone.

Equilibration time. Longer equilibration times give stronger binding species a greater chance to bind to the target, since weaker binding species more quickly dissociate from the target. In general, though, species with nanomolar dissociation constants or lower can be readily selected by allowing the reaction to equilibrate for 5 min or more. The authors usually allow up to 30 min for the binding reaction in order to permit slow folding or refolding steps in the presence of the target. However, longer equilibration times may not be possible for proteins that are inherently unstable or that themselves undergo slow, buffer- or temperature-induced conformational changes.

Dilution of binding buffer. Similarly, diluting the binding reaction by 10- to 20-fold just prior to filtration will favor the selection of RNA:protein complexes with low dissociation constants over RNA:protein complexes with higher dissociation constants. Baskerville et al. (1995) have successfully used this technique to select high affinity anti-Rex aptamers.

Amount and composition of wash. Increasing the number of times a filter is washed and the volume of the buffer used for the washes should preferentially increase the retention of high-affinity binding species relative to low-affinity and nonspecific binding species. It is generally recommended that the same buffer be used for selection and for wash steps, in order to avoid changing the conditions under which aptamers are selected. However, the stringency of the selection can potentially be manipulated by changing the buffer used for the wash steps. For example, if monovalent cation concentrations are limited in the binding buffer due to requirements for the stability or activity of a protein target, a separate wash buffer that contains a higher salt concentration can be used to challenge captured RNA:protein complexes.

Amplification kits

While the authors routinely utilize the kits described in this protocol, it goes without saying that many commercial kits are available for reverse transcription, the polymerase chain reaction, and in vitro transcription. However, the kits mentioned specifically in the protocols above have been found to be very useful in the Aptamer Selection Research Stream of the Freshman Research Initiative at the University of Texas at Austin. The students in this Stream have systematically assessed a variety of commercial kits with a variety of selection conditions. The kits were also evaluated with respect to cost, ease of use, robustness, and quality of results. For instance, the authors often utilize relatively inexpensive NEB *Taq* polymerase due to the quantity consumed over multiple rounds of selection. However, Platinum *Taq* (Invitrogen), AmpliTaq Gold (ABI), or Phusion (NEB) have been used to successfully amplify DNA when NEB *Taq* failed. It is reasonable to assert that if competent undergraduates can utilize these kits, then more experienced researchers should be able to obtain positive results with them.

The authors have also compared reverse transcriptases from Invitrogen (SuperScript), Applied Biosystems (MEGAScript), and Roche (Transcriptor). SuperScript II was found to be the most convenient to use. Lastly, the authors have tested a number of kits and components for transcription, including overexpressed and purified T7 RNA polymerase versus polymerases and kits from Invitrogen and Roche. Although relatively expensive for

our purposes, the AmpliScribe High Yield kit from Epicentre was chosen because of its consistent yield and robustness to template quality and incubation temperatures.

It should be noted that lot-to-lot variations are more common for "in house" enzyme preparations, and the yields are generally lower than those obtained with commercial kits. Troubleshooting homemade preparations can also be difficult relative to the technical support capabilities of a good reagent company. If users choose to prepare their own enzymes, a freshly expressed preparation should be fully tested for activity with controls, and then the same sample or aliquot should be utilized throughout the selection.

Parasites

Replication parasites differ from matrix-binding aptamers, but can interfere with the selection of target-binding aptamers in the same way. Reverse transcriptase, *Taq* polymerase, and T7 RNA polymerase all have some preference for which sequences they will copy or reproduce. These preferences are generally not obvious when constant-sequence nucleic acids are being synthesized. However, in selection experiments, many cycles of amplification are carried out, and differences in the rates of synthesis are also proportionately amplified, leading to the selection of sequences that have no function other than to replicate optimally. For example, during the polymerase chain reaction, if a primer designed to bind to a constant sequence region instead recognizes a partially complementary sequence within a random sequence region, it can bind and generate a smaller amplicon. The smaller amplicon will generally be amplified more quickly than the larger amplicon, and thus can potentially out-compete full-length species selected for binding function. Depending on the relative advantage of the replication parasite relative to an aptamer, even if the replication parasite is partially removed from the population during each selection step, enough molecules may remain to over-run the amplification reaction and displace the functionally selected aptamer. This is especially true if the amplification parasite also happens to be a filter-binding species. It is for this reason that DNA templates and/or RNA molecules should be size-selected in each round.

The nascent reproductive differences between nucleic acid species can be grossly amplified by amplification methods that allow continuous reproduction of the nucleic acids, such as isothermal amplification or 3SR (Guatelli et al., 1990). For example, Breaker and Joyce (1994) generated an extremely robust replication parasite, RNA Z, during a selection designed to generate catalytic variants of a group II intron. Similarly, the authors have generated replication parasites of isothermal amplification reactions from completely random sequence pools (K. Marshall, pers. comm.). Interestingly, these isothermal amplification parasites were actually larger than the initial RNA species and represented recombination events between individual members of the pool. Airborne copies of these replication parasites can readily "seed" isothermal amplification reactions and overrun pool molecules that are initially present in even million-fold excess. In this respect, the replication parasites of isothermal amplification reactions resemble the midi-variants or "monsters" of Qβ replicase amplification reactions, and are equally hard to vanquish, once established. It is for this reason that the authors strongly recommend the sometimes tedious but inherently faithful regime of reverse transcription, PCR, and in vitro transcription for the amplification of RNA pools. However, successful selections have been carried out that have relied upon isothermal amplification (see, for example, Breaker et al., 1994; Wright and Joyce, 1997; Wlotzka and McCaskill, 1997), and this admonition can most confidently be challenged if the starting pool is a partially randomized binding site or ribozyme. The reason is that isothermal amplification parasites are more likely to be found in or derived from a "deep random" pool than in a pool that centers on a given functional sequence.

Anticipated Results

Table 24.3.1 shows the progression of a selection carried out in the authors' lab against bFGF using an RNA pool with a 30-nucleotide-long randomized region. In order to evaluate the success of a selection experiment, it was necessary to compare the affinity of the selected pool versus the affinity of the unselected pool for the protein target (Support Protocol 3). When assaying the pool after a round of selection, it was necessary to validate the fraction of the pool that bound to the protein by including a no-protein control. If the accumulation of matrix-binding species had been evident, more stringent negative selections could have potentially been used to control or reduce their numbers.

The affinity of the RNA aptamer for the protein target cannot be anticipated. Affinity typically varies between micromolar and

sub-nanomolar, depending presumably on the makeup of the nucleotide pool and on the targeted protein. However, it might be worth mentioning that, of the first 100 selections carried out at two commercial entities using the technology—Gilead Sciences and NeXstar—just under 80% yield aptamers with affinities under 10^{-9} M (Brody et al., 1999). Recent innovations at Somalogic involving modified nucleotides have greatly increased both the rate of success and the affinities of selected aptamers (Zichi et al., 2008).

Time Considerations

The time required to go from one pool of selected DNA templates to the next is ~24 to 72 hr, depending on the researcher and the demands of the particular selection experiment. Minimally, a transcription reaction takes ~4 hr, and the ensuing DNase, heat-denaturation, and gel-purification steps can take another 2 to 3 hr. Elution for 8 to 10 hr yields an adequate amount of RNA to be used in the subsequent binding reaction. After precipitation and quantification of the RNA (1 hr), the preselection filtration, incubation with target, and selection steps can be performed in 2 hr. Elution of protein-RNA complexes, subsequent extractions, and another precipitation step take another 2 hr. The amount of time needed to see a DNA product varies according to the number of PCR cycles needed to amplify the pool to a certain amount, and that number is inversely related to the abundance of target-binding species that survived the selection. Nevertheless, the RT-PCR steps, followed by precipitation of the DNA templates that can be added to the transcription mix, should consume ~3 to 4 hr.

The amount of time it takes to carry out the entire selection is contingent upon the number of rounds needed to accumulate target-binding species. That number, in turn, varies depending upon the initial affinity of the unselected pool for the target and on the stringency with which each round of the selection is carried out. When additional steps such as radiolabeling and assaying unselected and selected pools are taken into account, an entire selection experiment can take up to 2 to 3 weeks. It is for this reason that the authors have recently developed automated methods for selection experiments (Cox et al., 1998) that can speed the entire process by an order of magnitude.

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Fickert, H., Betat, H., and Hahn, U. 2004. Selection of Aptamers. *In* Evolutionary Methods in Biotechnology: Clever Tricks for Directed Evolution (S. Brakmann and A. Schwienhorst, eds.) pp. 65-86. Wiley-VCH, Weinheim, Germany.

Stoltenburg et al., 2007. See above.

The above papers also describe protocols for the selection of aptamers via both filter immobilization and other separation methods.

Peptide Aptamers: Dominant "Genetic" Agents for Forward and Reverse Analysis of Cellular Processes

Peptide aptamers are a new class of dominant "genetic" agents that facilitate the analysis of cellular processes in diploid and genetically intractable organisms. They are defined as protein-based recognition agents that consist of a constrained combinatorial peptide library displayed on the surface of a scaffold protein. Peptide aptamers function in *trans*, interacting with and inactivating gene products without mutating the DNA that encodes them. Combinatorial libraries of peptide aptamers contain aptamers that, in principle, can interact with almost any gene product.

The dominant combinatorial nature of peptide aptamers makes them useful as genetic agents for the reverse and forward analysis of cellular processes. Reverse analysis with peptide aptamers involves isolating aptamers that interact with a specific protein and monitoring the resulting aptamer-induced phenotype. A two-hybrid system is used to screen combinatorial libraries of peptide aptamers for those aptamers that interact with a specific protein. The isolated aptamers are then expressed within an organism to identify the aptamer-induced phenotype. Forward analysis with peptide aptamers involves expressing combinatorial libraries of aptamers within an organism and screening for aptamer-induced variations in their phenotypes. The specific protein(s) targeted by the aptamers are identified using a two-hybrid system.

This unit describes methods to construct and use thioredoxin peptide aptamers as genetic agents for the analysis of cellular processes. The interaction trap two-hybrid system (*UNIT 20.1*) is used to isolate peptide aptamers that interact with specific proteins (reverse analysis) and to identify the proteins targeted by aptamers (forward analysis).

Basic Protocol 1 describes the construction of a combinatorial library of thioredoxin peptide aptamers. The peptide aptamers consist of a conformationally constrained twenty–amino acid peptide displayed from the active site of thioredoxin. The peptide aptamers are subcloned into one of the pJM yeast expression vectors shown in Figure 24.4.1, depending on whether they are used for reverse or forward analysis.

Basic Protocol 2 describes a yeast-based in vivo screening method to obtain peptide aptamers for reverse analysis of cellular processes. Combinatorial libraries of peptide aptamers are screened for interactions with a specific protein using the interaction trap two-hybrid system (*UNIT 20.1*). The peptide aptamer is expressed as a fusion to a transcription activation domain, referred to as the "prey." The target protein is expressed as a fusion to a LexA DNA binding domain, referred to as the "bait." DNA-binding sites for the LexA fusion protein are located upstream of the two reporter genes, *Leu2* (CD8) and *lacZ*. Interaction between a peptide aptamer prey and the bait protein are detected by activation of these reporter genes.

Basic Protocol 3 describes the use of the yeast mating interaction assay to evaluate the specificity of peptide aptamers. Haploid yeast exist in two mating types (**a** or α), where opposite mating types can mate to form diploids (**a**/α). The mating interaction assay detects aptamer/protein interactions by generating panels of aptamer preys in one mating type and panels of target bait proteins in the opposite mating type. Mating of the haploid strains forms diploid strains that carry both the bait and prey. Interactions between baits and preys are detected using the interaction trap reporters. The mating interaction assay

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allows aptamer specificity to be assessed against large arrays of different but related proteins and against mutants of the same protein.

Basic Protocol 4 describes an affinity maturation strategy for enhancing the affinity of peptide aptamers to their target proteins. PCR mutagenesis is used to introduce random mutations into the variable region of a peptide aptamer. Peptide aptamers with enhanced affinity are isolated using a modified version of the interaction trap that contains a more

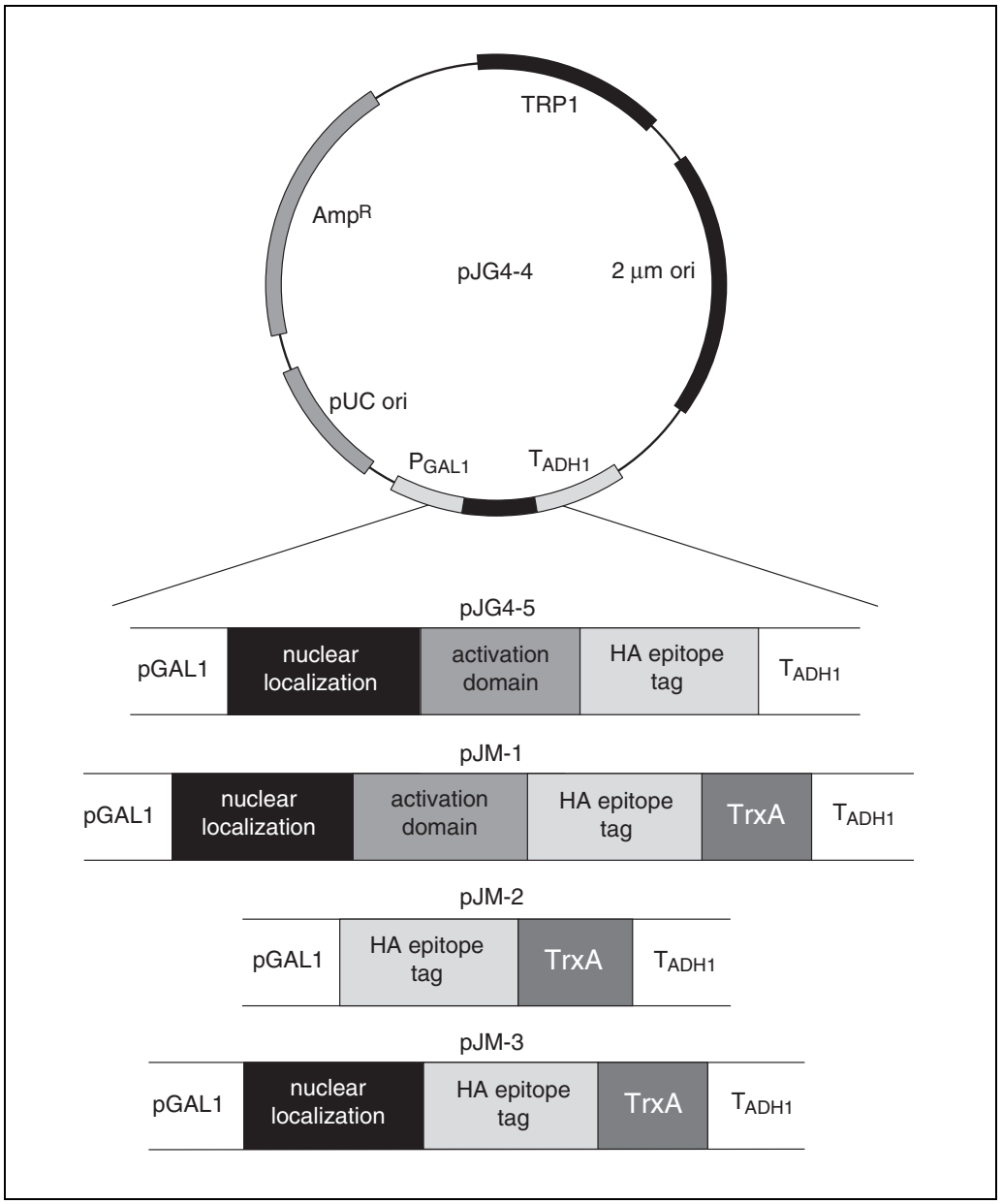

Figure 24.4.1 Expression vectors for interaction trap and genetic selection. pJG4-5 is the prey vector used in the interaction trap (*UNIT 20.1*). pJM-1 is the peptide aptamer prey vector. pJM-2 and pJM-3 are used in yeast genetic selections. These yeast-*E. coli* shuttle vectors are derivatives of pJG4-4 (Gyuris et al., 1993), and contain one of the following expression cassettes. pJG4-5: yeast GAL1 promoter (P_{GAL1}), SV40 nuclear localization signal, B42 activation domain, haemagglutinnin epitope tag, *Eco*RI and *Xho*I cloning site, and yeast ADH1 transcription terminator (T_{ADH1}) (Gyuris et al., 1993). See Figure 20.1.3 for a more detailed map of pJG4-5. pJM-1: P_{GAL1}, SV40 nuclear localization signal, B42 activation domain, haemagglutinnin epitope tag, *E. coli* thioredoxin (TrxA), and T_{ADH1} (Colas et al., 1996). pJM-2: P_{GAL1}, haemagglutinnin epitope tag, TrxA, and T_{ADH1} (Geyer et al., 1999). pJM-3: P_{GAL1}, SV40 nuclear localization signal, haemagglutinnin epitope tag, TrxA, and T_{ADH1} (Geyer et al., 1999).

stringent *lacZ* reporter. The stringency of the *lacZ* is increased by reducing the number of *LexA* operators upstream of the *lacZ* reporter gene.

Basic Protocol 5 describes a method to use peptide aptamers for the forward analysis of cellular processes. Combinatorial libraries of peptide aptamers are used as dominant genetic agents that randomly inhibit gene function. Forward analysis involves: (1) expressing combinatorial libraries of peptide aptamers in organisms, (2) isolating organisms that display aptamer-induced phenotypes, and (3) identifying peptide aptamer targets using the interaction trap.

CONSTRUCTION OF A COMBINATORIAL THIOREDOXIN PEPTIDE APTAMER LIBRARY

BASIC PROTOCOL 1

Combinatorial libraries of peptide aptamers are constructed by inserting a random twenty–amino acid peptide into the short disulfide-constrained loop (-CGPC-) in the active site of *E. coli* thioredoxin. The active site loop contains a unique *Rsr*II restriction site that allows the insertion of *Ava*II-cut DNA, which encodes for random amino acids. Random peptide libraries are constructed using twenty repeats of the codon NNK, where N is A, G, C, or T and K is G or C. Using G or C in the third position of the codon reduces the number of stop codons while maintaining codons for all twenty amino acids. Depending on the application, the random peptide libraries are subcloned into one of the pJM yeast expression vectors shown in Figure 24.4.1. pJM-1 is used in the interaction trap to generate peptide aptamers against specific proteins. pJM-2 and pJM-3 are used in genetic selections to produce aptamers that alter an organism's phenotype. All of the pJM vectors use the *gal1* promoter to control the expression of the peptide aptamers. The *gal1* promoter induces aptamer expression in the presence of galactose and represses expression in the presence of glucose. The resulting aptamer/thioredoxin vector is transformed into *E. coli* by electroporation (also see *UNIT 9.3* for electroporation techniques).

Materials

5 U/µl Klenow DNA polymerase and 10× reaction buffer (New England Biolabs)
5 mM 4dNTP mixture: 5 mM each dTTP, dATP, dGTP, and dCTP
10 U/µl *Ava*II and 2 U/µl *Rsr*II restriction enzymes and 10× reaction buffers (New England Biolabs)
10 mM Tris·Cl, pH 8 (*APPENDIX 2*)
Nondenaturing loading buffer (see recipe)
DNA elution buffer (see recipe)
Thioredoxin expression vector plasmid: pJM-1, pJM-2, or pJM-3 (Fig. 24.4.1)
10 U/µl calf intestinal alkaline phosphatase (CIP) and 10× reaction buffer (New England Biolabs)
2000 U/µl T4 DNA ligase and 10× reaction buffer (New England Biolabs)
QIAquick gel extraction kit (Qiagen)
Ultrapure water (sterile water for irrigation preferred; Fisher Scientific)
E. coli MC 1061 (Bio-Rad), electroporation competent (*UNIT 9.3*)
SOC medium (*UNIT 1.8*), prewarmed to 37°C
LB plates and liquid medium (*UNIT 1.1*) containing 50 µg/ml ampicillin
Large-scale plasmid preparation kit (various commercial sources, e.g., Qiagen; optional)

DNA synthesizer
16° and 95°C water baths
PCR purification column (e.g., Qiagen; optional)
Electroporator (e.g., Bio-Rad Gene Pulser) with 0.2-cm-gap electroporation cells

Additional reagents and equipment for DNA synthesis; phenol/chloroform extraction and ethanol precipitation (*UNIT 2.1A*); polyacrylamide gel electrophoresis (PAGE; *UNIT 2.7*); UV shadowing and elution of DNA (*UNIT 2.7*); UV spectroscopy (*APPENDIX 3D*) or ethidium bromide dot quantitation (*UNIT 2.6*); bacterial transformation (*UNIT 1.8*); and ethidium bromide/cesium chloride gradients (optional; *UNIT 2.4*)

NOTE: Activity units of enzymes are described for enzymes obtained from New England Biolabs. Other commercial sources can be used, but units should be confirmed.

Prepare random peptide DNA cassette

1. Prepare the following 91-base random oligonucleotide and 17-base primer using an automated DNA synthesizer. Dissolve oligonucleotides separately in water to a final concentration of 1 µg/µl.

   Oligonucleotide: 5′-GACTGACTGGTCCG$(NNK)_{20}$GGTCCTCAGTCAGTCAG-3′, where N is A, G, C, or T and K is G or C.

   Primer: 5′-CTGACTGACTGAGGACC-3′.

2. Add the following (in order) to a 1.5-ml microcentrifuge tube (final 890 µl):

   200 µg primer (10-fold excess)
   100 µg random oligonucleotide
   490 µl water
   100 µl 10× Klenow polymerase reaction buffer.

3. Anneal primer to random oligonucleotide by heating sample to 95°C in a water bath for 5 min. Slowly cool to room temperature (~30 min).

4. Add 90 µl of 5 mM 4dNTP mixture and 20 µl (100 U) Klenow polymerase and incubate 3 hr at 37°C.

5. Phenol/chloroform extract the mixture (*UNIT 2.1A*) and ethanol precipitate the DNA (*UNIT 2.1A*).

6. Dissolve DNA pellet in 0.8 ml water.

7. Add 100 µl of 10× *Ava*II reaction buffer and 100 µl (1000 U) *Ava*II. Incubate 4 hr at 37°C.

8. Repeat step 5.

9. Dissolve DNA pellet in 150 µl of 10 mM Tris·Cl, pH 8, and add 50 µl vol nondenaturing loading buffer.

10. Separate DNA on a preparative 10% nondenaturing polyacrylamide gel (*UNIT 2.7*).

11. Locate the DNA band in the gel by UV shadowing (*UNIT 2.7*) and cut out the DNA band.

12. Elute DNA from the gel by shaking in DNA elution buffer overnight (*UNIT 2.7*).

13. Ethanol precipitate the DNA and dissolve in 200 µl of 10 mM Tris·Cl, pH 8. Determine DNA concentration by UV spectroscopy (*APPENDIX 3D*), or estimate DNA concentration using ethidium bromide dot quantitation (*UNIT 2.6*).

Prepare thioredoxin expression vector

14. Choose one of the thioredoxin expression vectors (pJM) in Figure 24.4.1 and add 12 µg of the chosen vector to 420 µl sterile water.

15. Add 50 µl of 10× *Rsr*II reaction buffer and 30 µl (60 U) *Rsr*II. Incubate overnight at 37°C.

16. Dephosphorylate *Rsr*II-cut pJM vector by adding 10 µl (100 U) CIP and incubating 1 hr at 37°C.

17. Purify dephosphorylated, *Rsr*II-cut pJM vector using a commercially available PCR purification column or by phenol/chloroform extraction.

Ligate random peptide cassette in thioredoxin expression vector

18. Combine 8 µg DNA cassette (step 13) and 12 µg vector (step 17) in water to a total volume of 860 µl.

19. Add 100 µl of 10× T4 DNA ligase reaction buffer and 40 µl (80,000 U) T4 DNA ligase. Incubate 16 hr at 16°C.

20. Purify ligated DNA using a QIAquick gel extraction kit according to manufacturer's instructions. Elute DNA from the column using 30 µl ultrapure water.

    It is important to remove as much salt, buffer, and protein from the ligated DNA as possible prior to electroporation.

Electroporate ligated DNA

21. Thaw 350 µl electroporation-competent *E. coli* MC1061 on ice and add 30 µl purified ligated plasmid. Transfer mixture to a 0.2-cm-gap electroporation cell.

22. Electroporate using the following conditions: 2.5 kV, 200 Ω, and 25 µF.

23. Recover cells in 25 ml prewarmed SOC medium and incubate 1.5 hr at 37°C with gentle rocking.

24. Determine transformation efficiency by plating serial dilutions on LB plates containing 50 µg/ml ampicillin.

25. Transfer remaining cells to 1 liter LB liquid medium containing 50 µg/ml ampicillin and incubate overnight at 37°C.

26. Purify plasmid DNA using a commercially available large-scale plasmid preparation kit or using successive ethidium bromide/CsCl gradients (*UNIT 2.4*). Determine concentration and bring to 40 µg/ml for screening (Basic Protocol 2).

ISOLATION OF PEPTIDE APTAMERS FOR SPECIFIC PROTEINS USING THE INTERACTION TRAP TWO-HYBRID SYSTEM

BASIC PROTOCOL 2

The interaction trap two-hybrid system (Gyuris et al., 1993; *UNIT 20.1*) is an established method for screening proteins for interactions with genomic and cDNA libraries (reviewed by Bai and Elledge, 1996; Finley and Brent, 1997). The interaction trap can also be extended to screen combinatorial libraries of peptide aptamers for interactions with specific proteins (Yang et al., 1995; Colas et al., 1996). The interaction trap consists of the following parts: (1) a constitutively expressed target protein fused to a *LexA* DNA-binding domain, referred to as the "bait;" (2) a galactose-induced combinatorial library of thioredoxin peptide aptamers fused to an activation domain, referred to as the "prey;" and (3) *LexA*-operator-*leu2* and *LexA*-operator-*lacZ* reporter genes for detecting interactions between the peptide aptamer prey and target protein bait. The bait protein binds to the *LexA* operators upstream of the reporters, but does not activate transcription of the reporters. Interaction between a peptide aptamer prey and target protein bait is detected by activation of reporter genes in the presence of galactose and not in the presence of

glucose. Figure 20.1.2 illustrates the isolation of proteins that interact with specific targets using the interaction trap.

In the first part of this protocol, the bait plasmid (pBait) is constructed by inserting DNA that encodes for the target protein into the polylinker of pEG202, in frame with *LexA*. The chimeric *LexA*-bait fusion protein is constitutively expressed using the ADH1 promoter. It is transformed into the appropriate yeast strain (EGY48) by a standard lithium acetate transformation procedure (*UNIT 13.7*). To be useful in the interaction trap two-hybrid system, the bait proteins must enter the nucleus, bind to the *LexA* operators, and not self-activate the *leu2* and *lacZ* reporters. After construction, pBait is characterized using protocols described elsewhere (*UNIT 20.1*).

The pJM-1 peptide aptamer library is used to select aptamers that bind specific protein targets using the interaction trap. pJM-1 contains a thioredoxin aptamer fused to a nuclear localization signal, a transcription activation domain, and an epitope tag under the control of the *gal1* promoter. Peptide aptamer expression is induced in the presence of galactose and repressed in the presence of glucose. A high-efficiency lithium acetate transformation procedure (Gietz and Schiestl, 1995; outlined below) is used rather than the standard procedure (*UNIT 13.7*) to introduce the aptamer library into the yeast strain EGY48, which contains an integrated *LexA*–operator-*leu2* reporter gene, *LexA*-operator-*lacZ* reporter plasmid and a bait plasmid. Interactions between the bait protein and the peptide aptamer prey are initially detected on galactose plates that lack leucine. Galactose induces the expression of the peptide aptamer and the absence of leucine selects for peptide aptamer/bait protein interactions that activate the *leu2* reporter. Interactions are verified by subsequently testing for galactose-dependent growth on –Leu plates and galactose-dependent blue color on Xgal plates.

The lithium acetate transformation procedure used here typically yields 10^5 to 10^6 transformants per µg of plasmid DNA. The protocol should be optimized for individual strains to achieve maximum transformation efficiency. In particular, variables such as cell concentration and heat shock time need to be optimized. The highest transformation efficiencies are obtained with 1 µg plasmid DNA per 50 µl competent yeast cells and generally do not scale up with similar efficiencies. The protocol below is designed for the transformation of 50 µg of peptide aptamer library.

Materials

DNA encoding bait protein of interest
Plasmid DNA: pEG202 (Fig. 20.1.3), pSH18-34 (Fig. 24.4.2)
Yeast strain: EGY48 *ura3 trp1 his3 3LexA-operator-leu2*
Complete minimal (CM) dropout medium (*UNIT 13.1*) and plates supplemented with either 2% (w/v) glucose (Glu) *or* 2% (w/v) galactose and 1% (w/v) raffinose (Gal/Raf):
  Glu/CM –His,–Ura (10-cm plates and liquid medium)
  Glu/CM –His,–Ura,–Trp (10- and 15-cm plates)
  Glu/CM –His,–Ura,–Trp,–Leu (10-cm plates)
  Gal/Raf/CM –His,–Ura,–Trp (liquid medium)
  Gal/Raf/CM –His,–Ura,–Trp,–Leu (10- and 15-cm plates)
100 mM and 1 M lithium acetate, pH 7.5, filter sterilized
50% (w/v) polyethylene glycol, mol. wt. 3350 (PEG 3350; Sigma)
2 mg/ml single-stranded carrier DNA (sodium salt Type III from salmon testes; Sigma) TE buffer (*APPENDIX 2*)
40 µg/ml peptide aptamer library DNA (pJM-1 aptamer plasmid; see Basic Protocol 1)

2× glycerol storage solution: 65% (v/v) glycerol, 0.1 M $MgSO_4$, 25 mM Tris·Cl, pH 7.4 (*APPENDIX 2*)
10-cm Xgal plates (*UNIT 13.1*)
Glu/CM −His,−Ura,−Trp, Xgal
Gal/Raf/CM −His,−Ura,−Trp, Xgal
PCR primers for thioredoxin
30° and 42°C incubators or water baths

Additional reagents and equipment for subcloning DNA (*UNIT 3.16*); manipulating yeast (*UNIT 13.2*); lithium acetate yeast transformation (*UNIT 13.7*); characterizing bait plasmids (*UNIT 20.1*); determination of cell density (*UNIT 13.2*) and plating efficiency (*UNIT 20.1*); replica plating (*UNITS 1.3 & 13.2*); yeast plasmid preparation (*UNIT 13.11*); plasmid sequencing (*UNIT 7.3*); *E. coli* transformation (*UNIT 1.8*); agarose gel electrophoresis (*UNIT 2.5A*); and PCR (*UNIT 15.1*)

Construct bait plasmid (pBait)

1. Using standard subcloning techniques (*UNIT 3.16*), insert DNA that codes for the bait protein into the polylinker of pEG202 to create the bait plasmid (pBait).

2. Transform pBait and pSH18-34 (*lacZ* reporter) into the interaction trap selection strain (EGY48) by lithium acetate yeast transformation (*UNIT 13.7*).

3. Plate transformants on Glu/CM −His,−Ura plates and place in a 30°C incubator.

Characterize bait protein

4. Confirm that the bait protein does not self-activate the reporter genes by performing plate assays for *lacZ* activation and leucine requirement (*UNIT 20.1*).

   If the bait protein activates the leu2 and/or lacZ reporter genes, variations of the interaction trap that reduce reporter sensitivity should be tried. Yeast strains and/or plasmids containing less-sensitive leu2 and lacZ reporters reduce the background reporter output to reasonable levels. Yeast strains (Table 20.1.2) and plasmids (Fig. 24.4.2) with less sensitive reporters are described in UNIT 20.1. Truncating or separating the protein target can also eliminate transcription self-activation.

5. Confirm bait protein synthesis using the repression assay described in *UNIT 20.1*.

   Baits that do not repress the expression of β–galactosidase in the repression assay may not be expressed correctly or may be incapable of entering the nucleus. Expression of full-length baits can be verified by immunoblotting. If full-length baits are expressed, their entry into the nucleus can be facilitated by adding a nuclear localization signal (J. Kamens, unpub. observ.). See Table 20.1.1 for description of plasmid pJK202 (a bait vector that contains a nuclear localization signal).

Transform peptide aptamer library into pBait-containing yeast

6. Inoculate 20 ml Glu/CM −His,−Ura liquid medium with transformed EGY48 (step 3) and incubate overnight at 30°C with shaking.

7. Take an OD_{600} measurement and dilute to a concentration of 5×10^6 cells/ml in 250 ml Glu/CM −His,−Ura.

   An OD_{600} of 0.1 corresponds to ~3×10^6 cells/ml. This value should be confirmed for each yeast strain used (UNIT 13.2).

8. Incubate cells at 30°C with shaking until they reach an OD_{600} of 0.6 to 0.8 (~5 to 6 hr).

   This will yield enough yeast for 50 transformations.

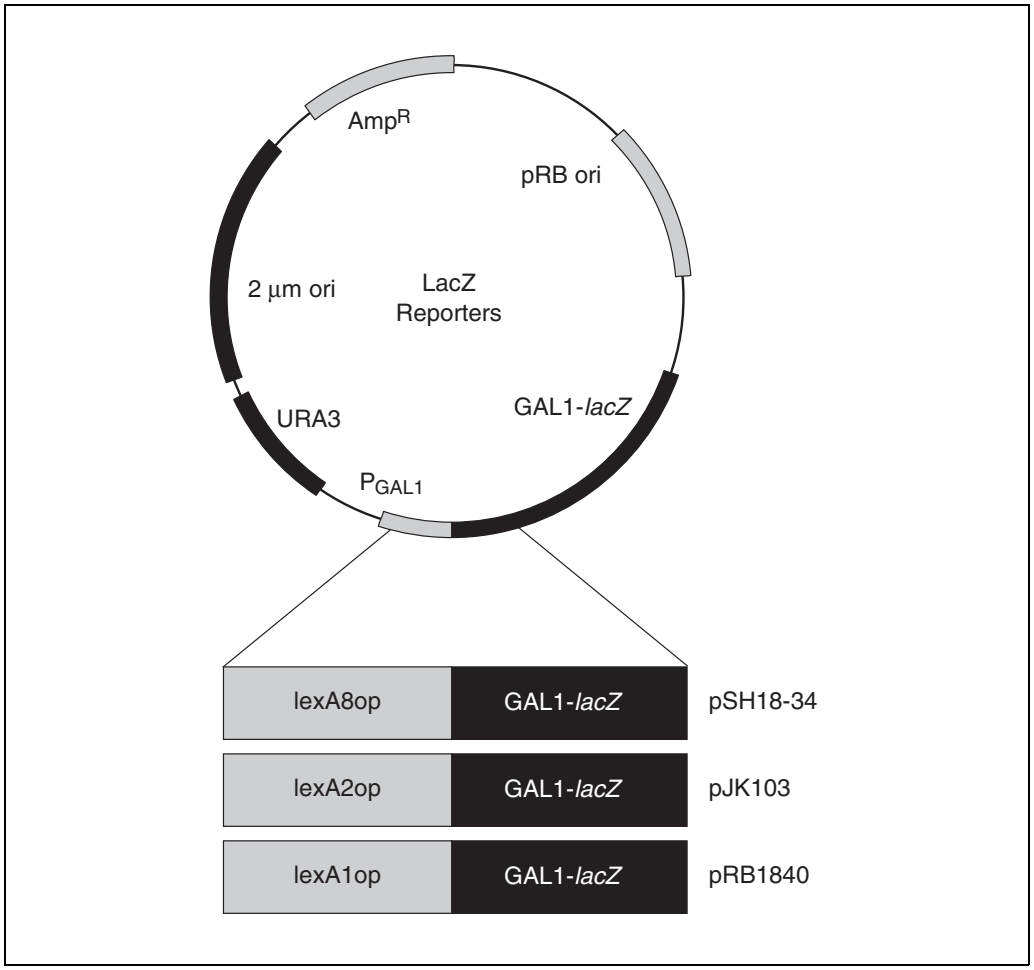

Figure 24.4.2 *lacZ* reporter plasmids. The *lacZ* reporter plasmids are derived from a plasmid that contains a wild-type GAL1 promoter fused to the *lacZ* gene (Yocum et al., 1984). *lacZ* reporters with different sensitivities are constructed by inserting different numbers of *lexA* operators into a plasmid (pLR1Δ1) that has the GAL1 upstream activating sequences (UAS_G) deleted (West et al., 1984). The *lacZ* reporters pSH18-34 (Gyuris et al., 1993), pJK103 (Kamens and Brent, 1991), and pRB1840 (Brent and Ptashne, 1985) contain eight, two, or one *lexA* operator(s). The sensitivity of the *lacZ* reporter decreases with the number of *lexA* operators.

9. Divide culture into five 50-ml conical centrifuge tubes and centrifuge 5 min at 3000 × *g*, room temperature.
10. Decant supernatant and resuspend each yeast pellet in 25 ml sterile water. Repeat centrifugation.
11. Decant supernatant and resuspend each yeast pellet in 1 ml of 100 mM lithium acetate. Transfer to a 1.5-ml microcentrifuge tube and pellet yeast by centrifuging 15 sec at 20,800 × *g*, room temperature.
12. Remove supernatant with a pipet and resuspend each yeast pellet in 350 µl of 100 mM lithium acetate (final volume ~500 µl).
13. Split the contents of each tube into ten 50-µl portions and pellet yeast by centrifuging 15 sec at 20,800 × *g*, room temperature.

14. Remove supernatant with a pipet and add the following ingredients to each sample in the order listed:

    240 µl 50% (w/v) PEG 3350
    36 µl 1 M lithium acetate
    50 µl 2 mg/ml single-stranded carrier DNA (100 µg)
    25 µl 40 µg/ml peptide aptamer library DNA (1 µg).

    Single-strand carrier DNA needs to be heated to 95°C for 5 min and cooled on ice prior to use.

15. Vortex the transformation mixture vigorously until the yeast pellet is completely resuspended and incubate 30 min at 30°C.

16. Heat shock 20 min at 42°C.

17. Pellet yeast by centrifuging 15 sec at 20,800 × g, room temperature.

18. Remove supernatant with a pipet and resuspend pellet in 500 µl sterile water.

19. Plate 48 transformations on individual 15-cm Glu/CM −His,−Ura,−Trp plates.

20. Plate 400 µl of the two remaining transformations on 15-cm Glu/CM −His,−Ura,−Trp plates.

21. Use the remaining 100 µl to determine the transformation efficiency. Perform a series of 10-fold dilutions in sterile water and plate on 10-cm Glu/CM −His,−Ura,−Trp plates.

22. Incubate 2 to 3 days at 30°C (until colonies are ~1 mm in diameter).

Pool transformants

23. Pool yeast from all 50 transformation plates (steps 19 and 20) in a 50-ml centrifuge tube.

    See UNIT 20.1 for protocol on scraping yeast from plates.

24. Add an equal volume of 2× glycerol storage solution to the pooled yeast cells. Divide into 1-ml aliquots and store at −70°C.

25. Determine the plating efficiency of the frozen aliquots as described in *UNIT 20.1*.

Screen for peptide aptamers that interact with target protein

26. Inoculate ten library equivalents of the peptide aptamer library in 2 ml Gal/Raf/CM −His,−Ura,−Trp liquid medium. Incubate 4 hr at 30°C with shaking.

    One library equivalent equals the total number of yeast transformants containing the peptide aptamer library, as determined in step 21.

27. Centrifuge 4 min at 3000 × *g*, room temperature.

28. Remove supernatant with a pipet and resuspend yeast in 1 ml sterile water.

29. Spread yeast at a density of 10^6 yeast cells/plate on 15-cm Gal/Raf/CM −His, −Ura, −Trp, −Leu plates.

30. Incubate at 30°C and monitor plates daily for growth.

31. Streak colonies onto 10-cm Glu/CM −His,−Ura,−Trp master plates. Incubate 1 to 2 days at 30°C.

32. Replica plate the master plates on the following indicator plates:

    Glu/CM –His,–Ura,–Trp,–Leu
    Gal/Raf/CM –His,–Ura,–Trp,–Leu
    Glu/CM –His,–Ura,–Trp, Xgal
    Gal/Raf/CM –His,–Ura,–Trp, Xgal.

33. Identify colonies that show galactose-dependent growth on –Leu plates and galactose-dependent blue color on Xgal plates.

Isolate peptide aptamers

34. Isolate the desired peptide aptamer expression plasmid (*UNIT 13.11*).

    The plasmid preparation will contain a mixture of the three plasmids used in the interaction trap (pJM-1 aptamer plasmid, pSH18-34, and pBait).

35. Use plasmids as templates for sequencing the peptide aptamer variable regions (*UNIT 7.3*).

36. To separate the aptamer plasmid from pBait and pSH18-34, transform *E. coli* (*UNIT 1.8*) and identify the appropriate transformants by PCR (*UNIT 15.1*) using primers that amplify thioredoxin.

    Colonies that contain the peptide aptamer will appear as a bright band on an ethidium bromide agarose gel (UNIT 2.5A) after 20 cycles of PCR. Colonies that do not contain the peptide aptamer will appear as a faint band that is 20 base pairs shorter than the aptamer. This shorter band is due to the presence of native E. coli thioredoxin.

BASIC PROTOCOL 3

DEFINING RECOGNITION SPECIFICITY WITH INTERACTION MATING

Interaction mating is a variation of the interaction trap. It allows interactions between large panels of proteins to be analyzed (Finley and Brent, 1994). Haploid yeast exist in one of two mating types (**a** or α). Haploid yeast that contain protein targets or related protein baits in one mating type and peptide aptamer preys in the opposite mating type can mate to form diploids that carry both the aptamers and their targets or related proteins. Interaction between the peptide aptamer prey and protein target bait is detected by the activation of two reporter genes: LexAop-LEU2 and LexAop-LacZ. Using the mating interaction assay, panels of related or mutated proteins can be assayed simultaneously for interactions with panels of peptide aptamers. See Figure 24.4.3 for schematic of the interaction mating assay.

Materials

Plasmid DNA: pBait(s) (see Basic Protocol 2), peptide aptamer preys (see Basic Protocol 2), pEG202 (Fig. 20.1.3), pJG4-5 (Fig. 24.4.1), pSH18-34 (Fig. 24.4.2)
Yeast strains:
  EGY42: *Matα ura3 trp1 his3 leu2*
  EGY48: *Mat***a** *ura3 trp1 his3 3LexA-operator-leu2)*
10-cm complete minimal (CM) dropout plates (*UNIT 13.1*) supplemented with either 2% (w/v) glucose (Glu) *or* 2% (w/v) galactose and 1% (w/v) raffinose (Gal/Raf):
  Glu/CM –Trp
  Glu/CM –His,–Ura
  Glu/CM –His,–Ura,–Trp,–Leu
  Gal/Raf/CM –His,–Ura,–Trp,–Leu
YPD plates (*UNIT 13.1*)
Xgal plates (*UNIT 13.1*):
  Glu/CM –His,–Ura,–Trp, Xgal
  Gal/Raf/CM –His,–Ura,–Trp, Xgal.

30°C incubator

Additional reagents and equipment for lithium acetate yeast transformation (*UNIT 13.7*) and replica plating (*UNITS 1.3 & 13.2*)

1. Transform individual peptide aptamer prey plasmids and a control plasmid (pJG4-5) into EGY48 (*Mat*α) using lithium acetate transformation (*UNIT 13.7*). Select transformants on 10-cm Glu/CM −Trp plates.

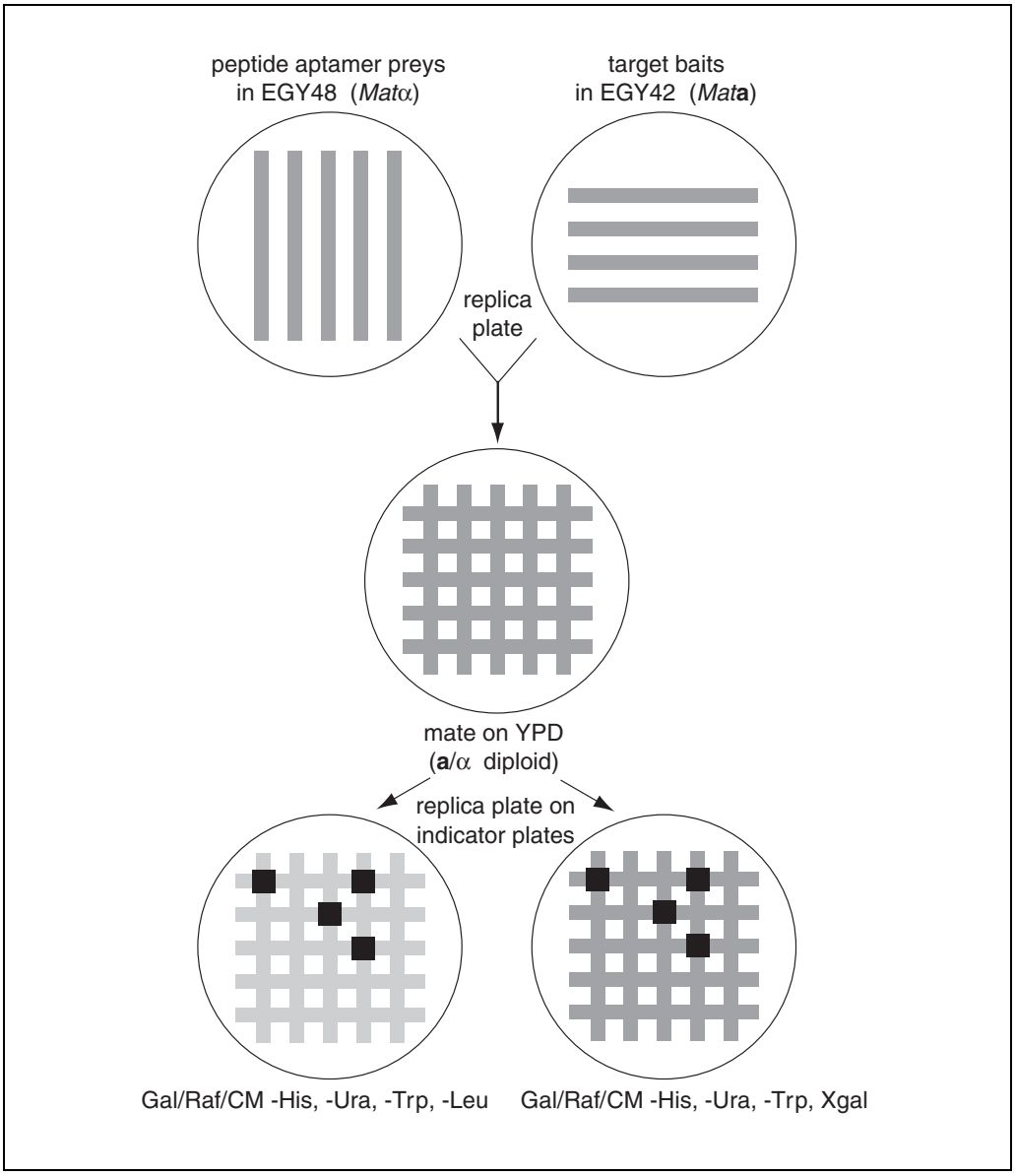

Figure 24.4.3 Mating interaction assay (Finley and Brent, 1994). Peptide aptamer preys in the yeast strain EGY48 (*Mat***a**) are streaked vertically on Glu/CM –Trp plates. Target protein baits and *lacZ* reporter (pSH18-34) in the yeast strain EGY42 (*Mat*α) are streaked horizontally on Glu/CM –His,–Ura plates. The yeast strains are replica plated perpendicular to each other on YPD plates. The haploid strains carrying the baits and preys mate where the two strains intersect, forming (**a**/α) diploids that contain the bait, prey, and *lacZ* reporter. The YPD plates are replica plated onto the following interaction detection plates: Glu/CM –His,–Ura,–Trp,–Leu; Gal/Raf/CM –His,–Ura,–Trp,–Leu; Glu/CM –His,–Ura,–Trp, Xgal; Gal/Raf/CM –His,–Ura,–Trp, Xgal. Interacting baits and prey display galactose-dependent growth and blue color on –Leu and Xgal plates, respectively.

2. Transform individual target protein baits (pBaits) with pSH18-34 (*lacZ* reporter) and a control plasmid (pEG202) with pSH18-34 into EGY42 (*Mat***a**). Select transformants on 10-cm Glu/CM −His,−Ura plates.
3. Streak, in parallel lines, individual peptide aptamers and their control prey strains on 10-cm Glu/CM −Trp plates.
4. Streak, in parallel lines, individual protein targets and their control bait strains on 10-cm Glu/CM −His,−Ura plates.
5. Incubate all plates overnight at 30°C.
6. Replica plate the protein target bait and peptide aptamer prey strains on the same replica velvet by first replica plating the bait strains and then replica plating the prey strains perpendicular to the baits (see Figure 24.4.3 for schematic).
7. Transfer the yeast imprint to a 10-cm YPD plate and incubate overnight at 30°C.
8. Replica plate the YPD plate onto a replica velvet. Transfer the yeast imprint to the following indicator plates:

   Glu/CM −His,−Ura,−Trp,−Leu
   Gal/Raf/CM −His,−Ura,−Trp,−Leu
   Glu/CM −His,−Ura,−Trp, Xgal
   Gal/Raf/CM −His,−Ura,−Trp, Xgal.

9. Analyze plates for mating.

   *Mating occurs at the intersection of the Matα and Mat***a** *strains. Diploid colonies should grow on the Xgal plates. Interactions between the peptide aptamer preys and protein target baits produce blue color on the galactose Xgal plates and growth on the galactose –Leu plates at the intersection of the strains.*

BASIC PROTOCOL 4

AFFINITY MATURATION OF PEPTIDE APTAMERS

The binding affinity between a peptide aptamer and its protein target can be improved by mutating the peptide aptamer variable region and reselecting for aptamers that bind the target protein using a more stringent interaction trap. In this protocol, peptide aptamers are mutated by random PCR mutagenesis as described by Cadwell and Joyce, 1994. Alternatively, degenerate oligonucleotides that code for the variable region and have varying degrees of randomness can be synthesized using an automated DNA synthesizer (*UNIT 2.11*). The stringency of the interaction trap selection is enhanced by decreasing the number of *LexA* operators upstream of the *lacZ* reporter gene. A series of *lacZ* reporter genes containing eight, two, and one *LexA* operator(s) (Brent and Ptashne, 1985) are used to select aptamers with increased affinity toward their targets.

Materials

5 U/µl *Taq* polymerase and 10× buffer (Life Technologies)
1 M $MgCl_2$
100 mM dATP
100 mM dGTP
100 mM dCTP
100 mM dTTP
20 µM primer 1: 5′-CCGCCGCCTGAATTCATGAGCGATAAAATTATTCAC-3′
20 µM primer 2: 5′-CGGGGCGATCATTTTGCACGGACC-3′
Plasmid DNA: peptide aptamer plasmid (see Basic Protocol 2), pBait (see Basic Protocol 2), pJM-1 (Fig. 24.4.1), pRB1840 (1-LexAop-LacZ reporter plasmid; Fig. 24.4.2), and pJK103 (Fig. 24.4.2)

Mg^{2+}/Mn^{2+} solution: 45 mM $MgCl_2$ and 5 mM $MnCl_2$
PCR purification column (optional; e.g., Qiagen)
Yeast strain: EGY48 *Mat***a** *ura3 trp1 his3 3LexA-operator-leu2*
Complete minimal (CM) dropout medium (*UNIT 13.1*) and plates supplemented with either 2% (w/v) glucose (Glu) *or* 2% (w/v) galactose and 1% (w/v) raffinose (Gal/Raf):
Glu/CM −His,−Ura (10-cm plates)
Glu/CM −His,−Ura,−Trp (10-cm plates)
Gal/Raf/CM −Ura,−His,−Trp (liquid medium)
Xgal plates (*UNIT 13.1*)
Glu/CM −His,−Ura,−Trp, Xgal (10-cm plates)
Gal/Raf/CM −His,−Ura,−Trp, Xgal (10- and 15-cm plates)

PCR tubes
Automated thermal cycler
30°C incubator

Additional reagents and equipment for agarose gel electrophoresis (optional; *UNIT 2.5A*), digesting and cloning peptide aptamer mutants (see Basic Protocol 1), lithium acetate yeast transformation (see Basic Protocol 2 and *UNIT 13.7*), determination of plating efficiency (*UNIT 20.1*), plasmid rescue (*UNIT 13.11*), and plasmid DNA sequencing (*UNIT 7.3*)

Mutagenize peptide aptamer variable region

1. Prepare PCR premixture (total 3.775 ml):

   500 µl 10× *Taq* polymerase buffer
   5 µl 1 M $MgCl_2$
   10 µl 100 mM dATP
   10 µl 100 mM dGTP
   50 µl 100 mM dCTP
   50 µl 100 mM dTTP
   125 µl 20 µM primer 1
   125 µl 20 µM primer 2
   2.9 ml water.

2. For each sample, add the following reagents to a PCR tube:

   12 µl water
   1 µl peptide aptamer expression vector
   10 µl Mg^{2+}/Mn^{2+} solution
   76 µl PCR premixture
   1 µl *Taq* polymerase (5 U).

3. Amplify the reaction using the following PCR reaction program:

4 cycles:	30 sec	95°C	(denaturation)
	1 min	55°C	(annealing)
	1 min	72°C	(extension).

4. Remove 13 µl reaction mixture and add to a new PCR tube containing:

   10 µl Mg^{2+}/Mn^{2+} solution
   76 µl PCR premixture
   1 µl *Taq* polymerase.

   Amplify using the same PCR program.

5. Repeat for a total of ten rounds of amplification.

6. Purify the PCR product with a commercially available PCR purification column or by agarose gel electrophoresis (*UNIT 2.5A*).

Construct mutagenized peptide aptamer expression vector

7. Digest purified PCR product with *Ava*II and subclone it into *Rsr*II-cut pJM-1 using standard subcloning techniques (*UNIT 3.16*). Electroporate the ligated product as described above (see Basic Protocol 1, steps 20 to 26).

Select mutagenized aptamers by the interaction trap

8. Transform EGY48 with pBait and pRB1840 by standard lithium acetate yeast transformation (*UNIT 13.7*). Select transformants on 10-cm Glu/CM −His,−Ura plates.
9. Using the high-efficiency lithium acetate procedure (see Basic Protocol 2, steps 6 to 22), transform 10 to 50 µg of mutagenized peptide aptamer library into EGY48 containing pBait and pRB1840. Select transformants on 10-cm Glu/CM −His,−Ura, −Trp plates.
10. Pool transformants and determine plating efficiency as described in *UNIT 20.1*.
11. Inoculate approximately five library equivalents in 1 ml Gal/Raf/CM −His, −Ura, −Trp liquid medium. Incubate 4 hr at 30°C with shaking.

    One library equivalent equals the total number of yeast transformants containing the peptide aptamer library as determined in step 9.

12. Centrifuge 4 min at 3000 × *g*, room temperature. Remove supernatant and resuspend yeast pellet in 1 ml sterile water.
13. Spread yeast on 15-cm Gal/Raf/CM −His,−Ura,−Trp, Xgal plates and incubate at 30°C until colonies appear (~2 days).
14. Streak blue colonies onto a 10-cm Glu/CM −His,−Ura,−Trp master plate and incubate 1 day at 30°C.
15. Replica plate the master plate onto 10-cm Gal/Raf/CM −His,−Ura,−Trp, Xgal and Glu/CM −His,−Ura,−Trp, Xgal plates.
16. Rescue plasmids (*UNIT 13.11*) from the galactose-dependent blue colonies and reintroduce (*UNIT 13.7*) the plasmids into the yeast strain EGY48 that contains pBait and pRB1840 to reconfirm the phenotype.
17. Rescue the plasmids from the galactose-dependent blue colonies and sequence (*UNIT 7.3*) the variable regions.

BASIC PROTOCOL 5

FORWARD ANALYSIS OF CELLULAR PROCESSES USING PEPTIDE APTAMERS

Combinatorial libraries of peptide aptamers can function as dominant agents for the forward analysis of cellular processes. Peptide aptamers function as “mutagens”, randomly inhibiting gene function and altering the phenotype of an organism. Forward analysis with peptide aptamers involves expressing combinatorial libraries in organisms and screening or selecting for aptamer-induced changes in their phenotypes. The peptide aptamer targets are subsequently identified using the interaction trap. The protein targets can be identified from panels of proteins using a mating interaction assay (Finley and Brent, 1994) or by screening for aptamer interactions against genomic or cDNA libraries using the interaction trap (*UNIT 20.1*). Currently, complete panels of proteins are not available for any organisms except yeast. As a result, panels of known proteins will need

to be combined with cDNA and genomic libraries of proteins to identify peptide aptamer targets.

The design of a genetic selection is beyond the scope of this protocol. A typical genetic selection requires the transformation of an organism selection strain with a peptide aptamer expression library containing 10^6 to 10^7 members. Peptide aptamers are expressed under the control of an inducible promoter, allowing the aptamer-induced phenotype to be confirmed by comparing the effects of the aptamer expression plasmid in the presence or absence of the inducer. The protocol described below for a genetic selection using yeast may be adapted to a variety of organisms.

Materials

Yeast strain for genetic selection
Peptide aptamer library: pJM-2 or pJM-3 (Basic Protocol 1; Fig. 24.4.1)
Complete minimal (CM) dropout liquid medium (*UNIT 13.1*) and plates supplemented with either 2% (w/v) glucose (Glu) *or* 2% (w/v) galactose and 1% (w/v) raffinose (Gal/Raf):
Glu/CM −Trp (10-cm plates)
Gal/Raf/CM −Trp (10-cm plates and liquid medium)
30°C incubator

Additional reagents and equipment for high-efficiency lithium acetate yeast transformation (see Basic Protocol 2), determination of plating efficiency (*UNIT 20.1*), isolation of plasmids (*UNIT 13.11*), plasmid DNA sequencing (*UNIT 7.3*), and target identification (see Support Protocol)

1. Transform 50 to 100 µg of the peptide aptamer library (in pJM-2 or pJM-3) into a yeast selection strain (10^6 to 10^7 transformants) using the high-efficiency lithium acetate transformation procedure (see Basic Protocol 2, steps 6 to 22). Plate transformants on 10-cm Glu/CM −Trp plates and incubate at 30°C until colonies are ~1 mm in diameter (~2 to 3 days).
2. Pool yeast cells and determine the plating efficiency as described in *UNIT 20.1*.
3. Inoculate ten library equivalents in 1 ml Gal/Raf/CM −Trp liquid medium. Incubate 4 hr at 30°C with shaking.

   One library equivalent equals the total number of transformants containing the peptide aptamer library as determined in step 1.
4. Centrifuge culture 4 min at 3000 × *g*, room temperature. Remove supernatant with a pipet and resuspend the yeast pellet in sterile water.
5. Plate yeast on 10-cm Gal/Raf/CM −Trp selection plates and incubate under selection conditions.
6. Streak positive colonies on Glu/CM −Trp master plates.
7. Confirm galactose-dependent phenotype by replicating master plate onto Glu/CM −Trp and Gal/Raf/CM −Trp plates and incubate under selection conditions.
8. Isolate peptide aptamer expression plasmids (pJM-2 or pJM-3) from the yeast colonies that show the galactose-dependent phenotype (*UNIT 13.11*).
9. Reconfirm the peptide aptamer phenotype by transforming the isolated plasmid into the selection strain and testing for galactose-dependent phenotype.
10. Isolate the peptide aptamer expression plasmids (*UNIT 13.11*) for sequencing (*UNIT 7.3*) and target identification (see Support Protocol).

SUPPORT PROTOCOL

IDENTIFICATION OF PEPTIDE APTAMER TARGETS

The protein targets of the genetically selected peptide aptamers (Basic Protocol 5) can be identified using the interaction mating assay (see Basic Protocol 3) or by interaction hunts against cDNA or genomic libraries (*UNIT 20.1*). Genomic and cDNA libraries are constructed as preys since they contain many sequences capable of activating transcription in the bait configuration. As such, the peptide aptamers need to be transferred to the bait plasmid pEG202 to identify their targets in these libraries. Protocols for constructing cDNA and genomic libraries can be found in *UNITS 5.7, 5.8A & 5.8B*.

Putative peptide aptamer targets identified with either mating interaction panels or hunts should be verified using genetic tests such as: (1) immunoprecipitation to confirm the aptamer interactions in vivo, (2) epistasis analysis to confirm that the aptamer functions in the same area as the target protein, or (3) comparison of the phenotype(s) caused by deletion and overexpression of target protein with the phenotype caused by the aptamer.

Materials

DNA encoding thioredoxin peptide aptamer (Basic Protocol 5)
Plasmid DNA: pEG202 (Fig. 20.1.3), pSH18-34 (Fig. 24.4.2), pJG4-5 (Fig. 24.4.1)
Yeast strains:
EGY42, *Mat*α *ura3 trp1 his3 leu2*
EGY48, *Mat***a** *ura3 trp1 his3 3LexA-operator-leu2*
Complete minimal (CM) dropout liquid medium (*UNIT 13.1*) and plates supplemented with either 2% (w/v) glucose (Glu) *or* 2% (w/v) galactose and 1% (w/v) raffinose (Gal/Raf):
Glu/CM −His,−Ura (10-cm plates)
Glu/CM −Trp (10-cm plates)
Prey library (see Table 20.1.3)

Additional reagents and equipment for PCR (*UNIT 15.1*), standard subcloning (*UNIT 3.16*), standard lithium acetate yeast transformation (*UNIT 13.7*), interaction mating (see Basic Protocol 3), interaction trap (*UNIT 20.1*)

Transfer peptide aptamers from pJM-2 or pJM-3 into pEG202

1. PCR amplify the DNA encoding the thioredoxin peptide aptamer using primers that contain restriction sites compatible with the polylinker of pEG202 and in frame with *LexA* (Fig. 20.1.3).
2. Using standard subcloning techniques (*UNIT 3.16*), insert the PCR product into pEG202 to create the peptide aptamer bait.
3. Transform the individual peptide aptamer baits and pSH18-34 (*lacZ* reporter) into EGY48 (*Mat*α) by standard lithium acetate yeast transformation (*UNIT 13.7*). At the same time transform a control plasmid (pEG202) and pSH18-34 into EGY48.
4. Select transformants on 10-cm Glu/CM −His,−Ura plates.

Identify targets

For mating interaction assay:

5a. Construct a panel of desired proteins by inserting coding regions of proteins into the polylinker of pJG4-5 (prey plasmid, Fig. 24.4.1).

6a. Transform prey plasmids and a control plasmid (pJG4-5) into EGY42 (*Mat***a**) by standard lithium acetate transformation. Select transformants on 10-cm Glu/CM −Trp plates.

7a. Mate strains containing peptide aptamer baits and target protein preys and score interactions as described (see Basic Protocol 3, steps 3 to 9).

For interaction trap library hunts:

5b. Transform strains containing individual peptide aptamers and pSH18-34 (step 3) with a library of genomic or cDNA preys. Follow the protocol in *UNIT 20.1* for transforming cDNA and genomic prey libraries.

6b. Select peptide aptamer target(s) using the interaction trap hunt protocol described in *UNIT 20.1*.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

DNA elution buffer

10 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
1 mM EDTA, pH 8 (*APPENDIX 2*)
50 mM NaCl
Store up to 1 year at room temperature

Nondenaturing loading buffer

50 mM Tris·Cl, pH 8 (*APPENDIX 2*)
50 mM EDTA, pH 8 (*APPENDIX 2*)
50% (v/v) glycerol
Store up to 1 year at 4°C

COMMENTARY

Background Information

Understanding cellular processes within organisms relies on forward and reverse genetic approaches to identify genetic network members and connections. In forward genetic analysis, genes are identified by isolating randomly generated mutants and mapping the genes responsible for their mutant phenotypes. Reverse genetic analysis, by contrast, involves mutating individual genes and monitoring the resulting phenotype. While both approaches are effective, they are difficult to perform, especially in diploid organisms. In diploid organisms, the identification of recessive mutations requires two generations of breeding to generate homozygotes. Consequently, genetic approaches requiring homozygous recessive mutations can only be fully applied to organisms with well-developed genetics such as phage, bacteria, yeast, *C. elegans*, and *Drosophila*.

Dominant agents that affect gene products in *trans*, instead of genes, have been developed to overcome problems associated with the analysis of recessive mutations in diploid organisms. A variety of dominant agents exist for the reverse analysis of cellular processes. These include: small molecule inhibitors (Mitchison, 1994), dominant negative proteins (Herskowitz, 1987), antibodies (Gorbsky et al., 1998), antisense RNA (Branch, 1998), ribozymes (Bramlage et al., 1998), and nucleic acid aptamers (*UNIT 24.3*; Thomas et al., 1997). These agents have improved the ability to analyze processes in diploid organisms; however, they too have limitations. For example, forward analysis requires large-scale generation of agents that are capable of inactivating the function of almost any gene product, but agents such as small molecule inhibitors and dominant negative proteins may not exist for all gene products. Similarly, although it should, in theory, be possible to generate agents such as antibodies, ribozymes, nucleic acid aptamers, and antisense RNA against almost any gene product, antibodies are not membrane permeable, and large-scale injection is tedious and impractical for most organisms. RNA agents are not very stable and it is difficult to predict sites on RNA that are exposed for inhibition by antisense RNA and ribozymes. Furthermore, agents that inhibit at the RNA level (antisense RNA and ribozymes) are affected by the stability of the protein target, which can affect the onset and/or the extent of the phenotype.

The development of combinatorial technologies for obtaining biomolecules with desired properties (*UNITS 24.2 & 24.3*; Ellington and Szostak, 1990; Scott and Smith, 1990) presents new avenues for generating "genetic" agents for characterizing genetically intractable organisms. This unit describes methods to construct combinatorial libraries of "genetic" agents referred to as peptide aptamers. Peptide aptamer libraries consist of scaffold proteins that display variable peptides constrained at both ends on their surface. They are designed to interact and interfere with the biological function of proteins. Peptide aptamers are well suited for analyzing cellular processes in diploid organisms because they act in *trans* to inhibit gene products without altering their encoding DNA. Moreover, because they are isolated from combinatorial libraries, peptide aptamers can in principle be generated to inactivate almost any gene product.

Design of intracellular peptide aptamers

Peptide aptamers are designed to interact with their protein targets through variable peptide regions displayed on the surface of a scaffold protein. To date, only a limited number of scaffold proteins have been used within organisms to display linear and constrained peptides. These include: *E. coli* thioredoxin (Colas et al., 1996), Gal4 activation domain (Yang et al., 1995), green fluorescent protein (Caponigro et al., 1998), and staphylococcal nuclease (Norman et al., 1999). A comparison of the binding constants of these aptamers shows that constrained variable regions can bind their targets between 100- and 10,000-fold better than linear peptides (Geyer and Brent, 2000). Unconstrained peptides are also known to be unstable in *E. coli* (Davidson and Sauer, 1994). Constrained peptide libraries are therefore the preferred method for displaying combinatorial peptide libraries for intracellular applications. When choosing aptamer scaffolds, they should also be small, stable, soluble, and expressed at high levels without toxicity. The scaffold should be tolerant to the addition of protein moieties such as localization sequences, epitope tags, and purification tags.

Basic Protocol 1 describes the construction of a peptide aptamer library using *E. coli* thioredoxin as the scaffold protein. Thioredoxin was first used as a scaffold protein for displaying peptides as fusions to flagellin on the surface of *E. coli* (Lu et al., 1995). Thioredoxin possesses many characteristics that make it an excellent scaffold for intracellular applications. Structural studies on thioredoxin reveal that its active site contains a 4–amino acid loop (-CGPC-) that is constrained by the two terminal cysteines (Katti et al., 1990). This loop is tolerant to peptide insertion (LaVallie et al., 1993) and provides a site for displaying variable peptides. Thioredoxin is a small (12 Kd) cytoplasmic protein that is nontoxic when expressed at high levels (LaVallie et al., 1993). Thioredoxin is often fused to proteins to enhance their solubility (LaVallie et al., 1993). This is a useful property for expressing random sequence libraries where many of the sequences may aggregate. Thioredoxin interacts with a variety of disulfide-containing protein substrates (Wetterauer et al., 1992), suggesting that it may also contribute to the binding interactions between peptide aptamers and their protein targets.

Reverse "genetic" analysis using peptide aptamers

Reverse genetic analysis using peptide aptamers involves isolating aptamers that interact with a specific gene product and monitoring the aptamer-induced phenotype. Peptide aptamers that interact with a chosen protein are selected using yeast two-hybrid systems or a variation thereof (Chien et al., 1991; Dalton and Treisman, 1992; Durfee et al., 1993; Gyuris et al., 1993; Vojtek et al., 1993). These systems share the following features: (1) a DNA-binding domain/target protein fusion, (2) a transcription activation domain/peptide aptamer fusion, and (3) reporter gene(s) to record interactions between the peptide aptamer and protein target (see *UNIT 20.1* for a detailed description of the yeast two-hybrid system).

Basic Protocol 2 describes the interaction trap two-hybrid system as a method for obtaining peptide aptamers that interact with a selected protein target. The interaction trap is an effective method for obtaining high-affinity peptide aptamers that bind specific proteins. Aptamers obtained using the interaction trap have dissociation constants greater than the 1 μM detection limit required to activate the interaction trap reporters (Estojak et al., 1995).

To date, the interaction trap has been used to isolate peptide aptamers against a variety of protein targets including Cdk2 (Colas et al., 1996), Ras (Xu et al., 1997), HIV-1 Rev (Cohen, 1998), and E2F (Fabbrizio et al., 1999). The dissociation and half-inhibitory constants of these aptamers range from 10^{-8} to 5×10^{-11} M.

An advantage of using the interaction trap to select peptide aptamers is that selection occurs in an intracellular environment. This increases the probability that the aptamers will retain their function when expressed in the appropriate organism. Moreover, aptamers isolated using the interaction trap function effectively under a variety of in vivo conditions such as cell cultures (Cohen et al., 1998; Fabbrizio et al., 1999) and in *Drosophila* (Kolonin and Finley, 1998).

Specificity of peptide aptamers

To be useful for genetic analysis, a peptide aptamer must interact specifically with its protein target. Peptide aptamer specificity can be evaluated by analyzing the aptamer's ability to interact with related target proteins using the interaction trap. Basic Protocol 3 describes the mating interaction assay, an extension of the interaction trap developed by Finley and Brent (1994) for determining the specificity of peptide aptamers against a large panel of related proteins. The interaction mating assay allows panels of individual aptamers to be simultaneously screened for interactions with panels of related target proteins. Using this method, Colas et al. (1996) determined the specificity of aptamers isolated against cyclin-dependent kinase 2 (Cdk2). The majority of aptamers tested were highly specific for Cdk2 and not other closely related kinases with one exception: some of the aptamers also interacted with the closely related kinase Cdk3. Their results demonstrate that aptamers can be generated against different epitopes on Cdk2, some of which are conserved between different members of the cyclin-dependent kinases.

The mating interaction assay is also used to determine the specific regions and/or amino acids that aptamers recognize on the target protein. For example, Cohen et al. (1998) showed that one of the aptamers isolated against Cdk2 (Colas et al., 1996) acts as a competitive inhibitor of the Cdk2-dependent phosphorylation of histone H1. Interaction mating with a panel of mutant Cdk2 proteins revealed that specific active site residues are required for aptamer binding, supporting the competitive inhibition mechanism. In summary, interaction mating assays using panels of related and mutated proteins can be used to classify both the specificity and binding interactions of different aptamers targeted to the same protein.

Affinity maturation

Peptide aptamer selections using the interaction trap are limited to screening ~10^6 to 10^7 unique aptamers per experiment. This is a small representation (~9×10^{-6}%) of the entire sequence space available to aptamers containing 20-mer variable regions (20^{20} possible sequences). In addition to the small sample size, many of the aptamers will contain stop codons within the variable region. As a result, it is likely that aptamers isolated using the interaction trap do not contain the optimal binding sequences for their target proteins.

Basic Protocol 4 describes a method to obtain aptamers with increased binding affinity. The protocol involves mutating the aptamer variable region and reselecting for binding to its target protein using an interaction trap that contains a more stringent reporter gene (Cohen, 1998; Colas et al., 2000). Mutations can be introduced using mutagenic PCR or by synthesizing degenerate oligonucleotides with varying degrees of randomness (see *UNIT 2.11* for a discussion on the construction of degenerate oligonucleotides). The stringency of the interaction trap is enhanced by reducing the number of *LexA* operators upstream of the reporters. The interaction trap in *UNIT 20.1* contains eight *LexA* operators in the *LexA-lacZ* (pJG4-5) and *LexA-leu2* (EGY48 strain) reporters. These reporters are capable of detecting interactions with dissociation constants of <1 μM (Estojak et al., 1995). Other *lacZ* reporters, developed by Brent and Ptashne (1985), contain only one (pRB1840) or two (pJK103) *LexA* operators (Fig. 24.4.2). These operators have lower affinity for the *LexA* DNA-binding domain and detect interactions with dissociation constants between 20 nM and <1 μM (Estojak et al., 1995).

The affinity maturation described in Basic Protocol 4 has been successfully used to enhance the affinity of aptamers isolated against Cdk2 (Cohen, 1998; Colas et al., 2000). The variable region of the anti-Cdk2 aptamer was mutated by PCR and reselected for binding to a LexA-Cdk2 fusion using the 1-*LexA*-operator *lacZ* reporter (pRB1840). Isolated aptamers all contained the same two amino acid substitutions. The dissociation constant of the mature aptamer was reduced to 5 nM, a 20-fold decrease from the starting aptamer ($K_d = 0.1$ μM).

Forward "genetic" analysis with peptide aptamers

Combinatorial libraries of peptide aptamers can function as dominant agents to randomly

Table 24.4.1 Degenerate Codons for Designing Combinatorial Peptide Libraries[a]

Codon[b]	Properties	Amino acids[c]	No. of codons	Stop codons
NNN	All 20 amino acids	A(4), C(2), D(2), E(2), F(2), G(4), H(2), I(3), K(2), L(6), M(1), N (2), P(4), Q(2), R(6), S(6), T(4), V(4), W(1), Y(2)	64	TAA(1), TAG(1), TGA(1)
NNS	All 20 amino acids	A(2), C(1), D(1), E(1), F(1), G(2), H(1), I(1), K(1), L(3), M(1), N(1), P(2), Q(1), R(3), S(3), T(2), V(2), W(1), Y(1)	32	TAG (1)
NNC	15 amino acids	A(1), C(1), D(1), F(1), G(1), H(1), I(1), L(1), N(1), P(1), R(1), S(2), T(1), V(1), Y(1)	16	None
NWW	Charged, hydrophobic	D(1), E(1), F(1), H(1), I(2), K(1), L(3), N(1), Q(1), V(2), Y(1)	16	TAA (1)
RVK	Charged, hydrophilic	A(2), D(1), E(1), G(2), K(1), N(1), R(1), S(1), T(2)	12	None
DVT	Hydrophilic	A(1), C(1), D(1), G(1), N(1), S(2), T(1), Y(1)	9	None
NVT	Charged, hydrophilic	A(1), C(1) , D(1), G(1), H(1), N(1), P(1), R(1), S(2), T(1), Y(1)	12	None
NNT	Mixed	A(1), C(1), D(1), F(1), G(1), H(1), I(1), L(1), N(1), P(1), R(1), S(2), T(1), V(1), Y(1)	16	None
VVC	Hydrophilic	A(1), D(1), G(1), H(1), N(1), P(1), R(1), S(1), T(1)	9	None
NTT	Hydrophobic	F(1), I(1), L(1), V(1)	4	None
RST	Small side chains	A(1), G(1), S(1), T(1)	4	None
TDK	Hydrophobic	C(1), F(1), L(1), W(1), Y(1)	6	TAG (1)

[a]Based on a table described by Sidhu and Weiss (2000).
[b]Abbreviations: D = A, G, T; K = G, T; N = A, G, C, T; R = A, G; S = C, G; V = A, C, G; W = A, T.
[c]Numbers in parentheses indicate the number of codons for each amino acid.

inactivate gene products without altering their genetic material. The forward analysis of cellular processes using peptide aptamers involves expressing libraries of peptide aptamers within cells and screening for aptamer-induced phenotypes. The protein(s) and protein interactions disrupted by the aptamers are then identified. Basic Protocol 5 describes methods for performing forward analysis of cellular processes in yeast. Methods are also described for identifying peptide aptamer target(s) using interaction trap hunts with genomic or cDNA libraries or by mating interaction assays using protein panels (Support Protocol). Combining aptamer library screening with interaction trap hunts and mating interaction assays provides a new strategy for analyzing processes in diploid organisms and in multicopy gene phenotypes.

Peptide aptamers have been used for the forward analysis of phenotypes in yeast (Caponigro et al., 1998; Geyer et al., 1999; Norman et al., 1999) and bacteria (Blum et al., 2000). In yeast, peptide aptamers were isolated that inhibited mating pheromone response (Caponigro et al., 1998; Geyer et al., 1999; Norman et al., 1999) and spindle checkpoint (Norman et al., 1999) signal transduction pathways. In bacteria, peptide aptamers were isolated that specifically inhibited thymidylate synthase or that caused growth inhibition (Blum et al., 2000). The peptide aptamer targets for forward analysis in yeast were identified using yeast two-hybrid systems. Mating interaction assays identified protein targets from panels of proteins known to be involved in the yeast pheromone response pathway (Caponigro et al., 1998; Geyer et al., 1999) or from large

panels of proteins containing almost all of the proteins in the yeast genome (Norman et al., 1999). Peptide aptamer targets were also identified using interaction trap hunts against a partial-coverage yeast genomic library (Geyer et al., 1999). Interestingly, the peptide aptamer targets identified with the mating interaction assay were not obtained with the interaction trap hunt using the partial-coverage yeast genomic library (Geyer et al., 1999). The inability of the genomic library screen to identify aptamer targets is partly due to the representation of targets in the partial-coverage library. Nevertheless, the results demonstrate a better success rate for identifying aptamer targets using mating interaction assays with arrayed panels of protein targets. Mating interaction assays have the following advantages: (1) they present protein targets as fully normalized libraries, (2) they allow reporter outputs that result from interactions to be directly compared with outputs caused by the bait alone, and (3) they allow the detection of interaction strengths independent of the differences in plating efficiencies caused by differential reporter activation (Estojak et al., 1995). Currently, protein panels that cover an organism's entire proteome are not commercially available. Consequently, the identification of targets for peptide aptamers isolated using genetic screens will consist of limited panels of known proteins complimented with cDNA or genomic libraries.

Inhibitory mechanisms of peptide aptamers

Peptide aptamers inhibit protein function by a variety of mechanisms. For example, peptide aptamers can bind to protein targets and disrupt their interactions with other proteins. They can disrupt protein interactions within cells (Xu et al., pers. comm.) and in two-hybrid assays (Geyer et al., 1999), and they can inhibit enzymes by competing with their substrates for active site binding (Cohen et al., 1998). In addition to disrupting protein interactions, peptide aptamers can also inhibit protein function by mislocalizing protein targets. Peptide aptamers modified with a localization signal can transport their target proteins into various cellular compartments (Colas et al., 2000). Peptide aptamers fused to catalytic domains can also direct the substrate specificity of enzymes. They can be used to localize enzyme activities to specific protein targets (Colas et al., 2000) or locations in the cell.

Peptide aptamers are particularly useful for the analysis of genetic networks since they can disrupt specific interactions with protein targets that have multiple protein interactions (Geyer et al., 1999). This allows phenotypes caused by the disruption of individual interactions in a network to be observed, while leaving other interactions in the same network intact. Peptide aptamers can be isolated against allelic variants of proteins (Xu et al., 1997). Their high specificity can be used to functionally characterize variants of polymorphic proteins. In addition, controlling their expression using inducible promoters allows the penetrance and timing of the aptamer-induced phenotype to be varied. Finally, performing genetic selections with peptide aptamers targeted to different locations in the cell can provide information on the cellular location of the target protein.

Together, these properties point to the many ways in which peptide aptamers can be used to analyze cellular processes. The successful use of peptide aptamers in the reverse analysis of processes in cell cultures and in *Drosophila*, and in the forward analysis of processes in yeast, illustrates their potential as "genetic" agents in the analysis of genetically intractable organisms.

Critical Parameters and Troubleshooting

Peptide aptamer libraries

The first critical parameter to consider is the method for synthesizing peptide aptamer libraries. Preferably, peptide aptamer libraries are constructed to minimize the amount of stop codons while maintaining amino acid diversity. In general, two methods of automated DNA synthesis are used to generate DNA templates that code for combinatorial peptide libraries. The first method generates DNA templates by sequentially coupling mixtures of the four-nucleotide phosphoramidites. The second method generates DNA templates by sequentially coupling mixtures of codons.

The sequential nucleotide incorporation method uses completely random or biased mixtures of nucleosides to construct DNA templates. DNA templates constructed using equimolar mixtures of the four-nucleotide phosphoramidites contain all 64 possible codons, including 41 redundant codons and three stop codons. The completely random libraries are biased for amino acids encoded by multiple codons. In addition, the presence of stop codons produces truncated aptamers at a frequency of $3n/64$, where n is the length of the peptide library. The sequential nucleotide incorporation method is improved by restricting the nu-

cleotides that are incorporated at the third position in the codon (see Table 24.4.1 for examples of degenerate codons). The third position of a codon is responsible for most of the redundancy in the genetic code. DNA templates that contain all four nucleosides in the first two positions of the codon and only G or C at the third position consist of 32 codons, which code for 20 amino acids and one stop codon. Codons limited to G or C at the third position are biased for amino acids that are coded by multiple codons. However, the frequency of a stop codon is reduced to *n*/32, where *n* is the length of the peptide. The presence of stop codons in a completely random or third position–biased library limits the complexity that is obtainable with long combinatorial peptide libraries. The construction of longer peptide libraries requires the ligation of shorter DNA templates that are prescreened to eliminate sequences that contain stop codons (Cho et al., 2000). Alternatively, combinatorial peptide libraries can be constructed that contain no stop codons, but with reduced amino acid diversity. Table 24.4.1 provides examples of degenerate codons that can be used to design peptide libraries.

The sequential codon incorporation method is used to generate DNA templates that contain 20 amino acids and no stop codons. Three strategies are used to generate codons. The first strategy involves sequentially coupling individual nucleotide phosphoramidites to generate 20 codons each of which is on a separate column (Lam et al., 1991). The beads from each column are subsequently mixed together and repacked into new columns for the synthesis of the next codon. The second strategy involves the synthesis of 20 trinucleotide phosphoramidite codons (Virnekas et al., 1994). Combinatorial peptide libraries are synthesized by coupling random or biased mixtures of the codon phosphoramidites. The third strategy combines aspects of the first two strategies and involves sequentially coupling either an A, G, C, or T phosphoramidite followed by a specific dinucleotide phosphoramidite to complete the codon (Neuner et al., 1998). After the completion of each codon, the beads from the columns are mixed and repacked into new columns for the synthesis of the next codon. The advantage of the codon incorporation method is that it generates unbiased libraries without stop codons. However, there are drawbacks to this method. For example, the bead splitting can become extremely laborious for long peptides. Also, the synthesis of dinucleotide and trinucleotide phosphoramidites is not trivial, and these phosphoramidites are not currently commercially available.

Once the combinatorial peptide libraries are constructed and inserted into the scaffold protein, they need to be transformed into *E. coli* and amplified. Electroporation is the most efficient method for transforming high-diversity libraries into *E. coli*. DNA uptake by *E. coli* is maximized under conditions of high field strength and low current flow (see Sidhu and Weiss, 2000, for conditions to maximize transformation efficiency in *E. coli*). To reduce the current flow, the conducting species must be removed from the DNA using affinity purification columns.

The number of peptide aptamers that can be screened is generally limited by the transformation efficiencies of the organism used in the selection. In yeast, the highest transformation efficiencies are obtained using the lithium acetate transformation protocol developed by Geitz and Schiestl (1995). The diversity of peptide aptamer libraries in yeast are limited to ~10^6 to 10^7 unique aptamers. This is much lower than the 10^9 to 10^{10} libraries typically obtained in *E. coli*. Particular care should be taken to optimize the transformation efficiencies in yeast or other selected organisms. To obtain optimal transformation in yeast, it is important to perform trial transformations to optimize parameters such as heat shock time and cell density.

Screening peptide aptamers

A second critical parameter is the spontaneous reversion rate in the screen used to isolate the peptide aptamers. *UNIT 20.1* discusses critical parameters that should be taken into account when selecting peptide aptamers against specific proteins using the interaction trap. False positives that occur in either the interaction trap or other genetic screens can be eliminated more efficiently using peptide aptamers that are expressed under the control of an inducible promoter.

Identifying protein targets

A third critical parameter is the identification of proteins targeted by peptide aptamers that have been isolated based on their ability to disrupt cellular processes. In general, peptide aptamer targets are more reliably obtained from panels of known proteins rather than from genomic or cDNA libraries. Once putative peptide aptamer targets have been identified using interaction trap hunts and mating interaction assays, it is important to verify these targets

using other means. For example, immunoprecipitation can be used to confirm that aptamers form complexes with their targets under in vivo conditions. Genetic tests such as epistasis analysis can be used to identify the location of the aptamers relative to a known protein. Peptide aptamer targets can be deleted or overexpressed and the resulting phenotype compared to the aptamer-induced phenotype. Similarly, whole-genome transcript arrays can test whether aptamers cause the same response as known inhibitors or mutations.

Anticipated Results

In general, approximately one out of every 10^5 peptide aptamers screened using the interaction trap interacts with a given target protein (Colas et al., 1996; Xu et al., 1997; Fabbrizio et al., 1999). Based on results using the yeast pheromone response pathway as a model process, approximately one out of every 10^5 to 10^6 peptide aptamers can inhibit a cellular process (Geyer et al., 1999). These results apply to 20mer combinatorial peptide libraries displayed on the surface of *E. coli* thioredoxin.

Time Considerations

Basic Protocol 1: Construction of the thioredoxin peptide aptamer library and its subsequent electroporation and amplification in *E. coli* will take ~1 week.

Basic Protocol 2: Isolation of peptide aptamers that interact with a specific bait protein takes ~3-4 weeks. The bait plasmid (pBait) and the pJM-1 peptide aptamer library are constructed during the first week. During the second week the pBait and the *lacZ* reporter plasmid (pSH18-34) are transformed into EGY48. The bait protein is also assayed to determine if it self-activates the reporter genes. During the third week the peptide aptamer library is transformed into EGY48 that contains pBait and pSH18-34. The aptamers are screened for their ability to interact with the bait protein and putative interacting aptamers are obtained. A fourth week is required to isolate the aptamer plasmids from the yeast and sequence their variable regions.

Basic Protocol 3: Determination of the peptide aptamer specificity using interaction mating takes ~1-2 weeks. The time required to construct the bait proteins, which will be used to evaluate the aptamer specificity, varies depending on the number of baits chosen and difficulty in cloning the baits. Once the bait proteins are constructed it takes ~1 week to transform both the baits and *lacZ* reporter into EGY42 and the peptide aptamer preys into EGY48. Mating EGY48 with EGY42 and scoring interactions between the peptide aptamer preys and baits takes an additional week.

Basic Protocol 4: Affinity maturation of peptide aptamers takes ~3-4 weeks. The mutagenesis of the peptide aptamer and subsequent cloning into pJM-1 (prey vector) takes ~1 week. Isolation of mutant aptamers that interact with the bait protein using the interaction trap with a more stringent lacZ reporter takes 3 weeks as described above in Basic Protocol 2.

Basic Protocol 5: Construction of the thioredoxin peptide aptamer library (pJM-2 or pJM-3) takes ~1 week as described in Basic Protocol 1. The time required to isolate peptide aptamers that disrupt a cellular process varies depending on the organism and selection or screen used. Before the targets of the peptide aptamers can be identified, it is necessary to transfer the thioredoxin peptide aptamers from the expression vector used in the screen (pJM-2 or pJM-3) to the bait plasmid (pEG202). This transfer takes ~1 week. Identification of the peptide aptamer target(s) using the interaction trap mating (Basic Protocol 2) or cDNA or genomic library (*UNIT 20.1*) hunts takes ~4 weeks.

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Colas et al., 1996. See above.

First article to describe the interaction trap as a method to isolate thioredoxin peptide aptamers against a specific protein (Cdk2).

Geyer et al., 1999. See above.

Describes the use of thioredoxin peptide aptamers for the forward analysis of the pheromone response pathway in yeast. Peptide aptamers were isolated that disrupt the pathway. Peptide aptamer targets were identified using mating interaction assays that contained panels of known proteins and by using interaction trap hunts against a yeast genomic library.

Gyuris et al., 1993. See above.

Initial description of the interaction trap.

Finley and Brent, 1994. See above.

Initial description of the mating interaction assay.

Kolonin and Finley, 1998. See above.

Describes the reverse analysis of a cellular process in Drosophila using peptide aptamers that bind to Drosophila Cdks.

Lu et al., 1995. See above.

First study to use E. coli thioredoxin as a scaffold for displaying combinatorial libraries of peptides.

Norman et al., 1999. See above.

Describes the use of staphylococcal nuclease peptide aptamers for the forward analysis of the yeast pheromone response and the spindle checkpoint signal transduction pathways. Peptide aptamers are characterized by transcript arrays and by two-hybrid analysis using a protein panel containing almost all of the proteins in the yeast genome.

Sidhu and Weiss, 2000. See above

Review article that describes strategies for designing combinatorial peptide libraries and efficient methods for transforming E. coli.

Internet Resources

http://www.umanitoba.ca/faculties/medicine/units/biochem/gietz/Trafo.html

Web site that describes efficient protocols for transforming yeast.

See UNIT 20.1 for Internet resources related to the interaction trap.

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Protein Selection Using mRNA Display

mRNA display is an in vitro technique that may be used to search natural or synthetic DNA libraries for the functional proteins and peptides they encode. mRNA-displayed proteins are constructs in which a protein is covalently attached to the RNA that encodes it. This direct covalent association of phenotype (protein) and genotype (RNA) renders the protein directly amplifiable. This in turn allows successive cycles of selection, enrichment, and, optionally, mutagenesis, to be performed upon libraries of displayed proteins. At the end of this process, functional sequences will dominate the library; cloning and sequencing will reveal the identity of the selected functional proteins. mRNA display allows new functional proteins to be discovered without resorting to protein design.

mRNA-displayed proteins are generated by the in vitro translation of mRNA display templates which are mRNA molecules 3′-terminated in puromycin (Fig. 24.5.1). Puromycin is a translation inhibitor that is able to enter the ribosome during translation and form a stable covalent bond with the nascent protein. This allows a stable covalent linkage to be formed between the mRNA display template and the protein it encodes, resulting in an mRNA-displayed protein (Fig. 24.5.2).

STRATEGIC PLANNING

The first issue that needs to be addressed when embarking upon protein selection using mRNA display is the design and construction of the library at the DNA level. If the goal of the selection is the "improvement" of an existing protein aptamer or enzyme, then the starting point for the selection will be the DNA sequence encoding this protein. If the goal of the selection is to discover a new class of protein aptamers or enzymes, then the starting point for the selection will be a DNA sequence in which some or many of the positions

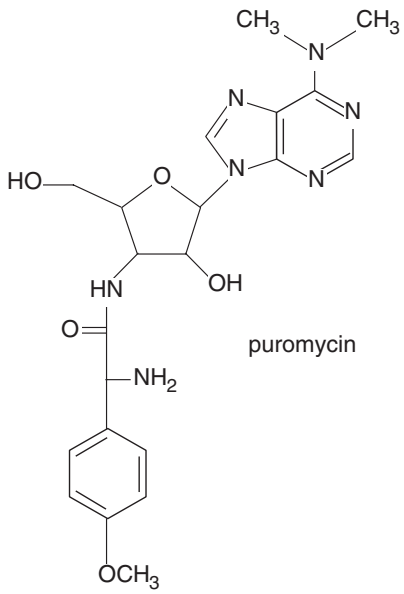

Figure 24.5.1 Puromycin is an antibiotic that functions by inhibiting translation. The molecular structure of puromycin resembles the acceptor arm of an amino-acylated tRNA. Puromycin is a nucleotide-amino acid chimera and ultimately forms the nucleic acid-protein junction in the mRNA displayed protein.

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are randomized. In either case, the DNA library may originate from a fixed natural sequence (clone) and subsequently be randomized by some process such as mutagenic PCR (Cadwell and Joyce, 1992) or DNA shuffling (Stemmer, 1994). Alternatively, the DNA library may be synthetic, in which case it can be synthesized as a fixed sequence and treated as above, or it may be synthesized in a high-diversity form directly, using mixtures of nucleotide phosphoramidites on a DNA synthesizer. In a third approach, the DNA may be isolated from a natural high-diversity source, either cDNA derived from biological mRNA or genomic DNA using a diverse mixture of PCR primers, or DNA sampled directly from the environment originating from a multitude of uncultured organisms. In all of these approaches, the DNA library will ultimately need to encode terminal constant regions to permit PCR amplification. These may also encode protein affinity tags that will facilitate the purification of the resulting displayed proteins. It is also desirable to encode restriction sites close to the random-constant sequence boundary to enable these constant regions to be changed should reengineering of the library be necessary.

If the DNA library is to be synthesized in more than one piece, then a strategy of restriction and ligation of different DNA cassettes needs to be designed. This strategy ultimately

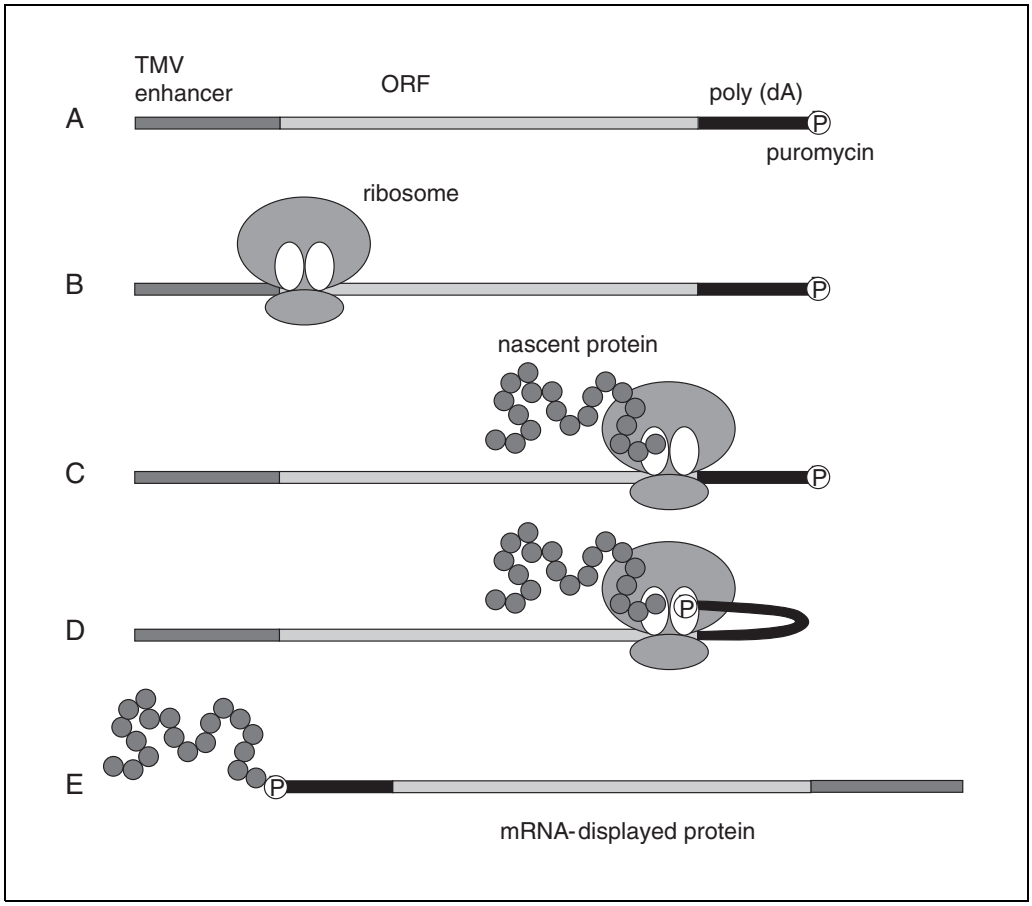


Figure 24.5.2 mRNA-displayed protein formation. (**A**) The mRNA display template consists of a Tobacco Mosaic Virus (TMV) translation enhancer sequence followed by the open reading frame encoded in RNA. This is followed by poly(dA) that is 3′-terminated with puromycin. (**B**) The ribosome initiating the translation of the mRNA display template. (**C**) The ribosome pausing at the RNA-DNA junction of the mRNA display template after it has translated the mRNA display template into protein. (**D**) The puromycin attached to the 3′-terminus of the mRNA display template entering the A site of the ribosome and forming a stable amide bond with the nascent protein. (**E**) The mRNA display template displaying the protein that it encodes after the ribosome has been released during purification.

yields the full-length library, and additionally offers the opportunities of purification and amplification of the individual DNA cassettes before they are ligated together. Amplification of the cassettes at this stage can greatly increase the library diversity in a combinatorial sense. Purification of the cassettes at this stage can decrease the proportion of cassettes that contain deletions, insertions, and stop codons. This "preselection" strategy can greatly increase the effective diversity of the DNA library since the proportion of resultant mRNA display templates, which are able to display frameshift-free proteins, will also increase in a combinatorial fashion once the DNA cassettes are ligated together. The steps in a preselection strategy are shown in Figure 24.5.3, and a detailed description is given in Cho et al. (2000).

Once the library has been designed and synthesized, the translation conditions need to be optimized for the formation of displayed proteins, and the purification strategy also needs to be optimized and subsequently piloted in a serial manner. However, the most important part of the strategic planning phase of the project is the design of the selection strategy. The first selection step needs to be designed to retain as many as possible of the functional displayed proteins that are present in the library, while discarding the great majority of those that are not functional. In this manner, the diversity of the library is taken advantage of to the maximal extent, but the diversity of the library is sufficiently reduced that the first amplification step is able to give several copies of the selected proteins for input into the next selection step. Subsequent to the first amplification step, the selection steps need to be designed to have the maximal possible reasonable discrimination between mRNA displayed proteins that exhibit the function of interest and those that do not. If at all possible, the extent of this discrimination should be assayed with positive and negative controls.

The steps within a single round of selection and amplification are shown in Figure 24.5.4.

Library Design

Library synthesis and preselection

A synthetic DNA library encoding a short open reading frame (ORF; up to ~35 amino acids) may be synthesized as a single oligonucleotide. Longer libraries of ORFs will need to be synthesized in two or more DNA cassettes that are then ligated together. Synthetic DNA has a deletion rate of ~0.5% and random regions will contain stop codons. Deletions will cause parts of the resultant proteins to be out of frame, and stop codons will prevent translated proteins from being displayed upon the mRNA that encodes them. For example, if the target ORF is 100 amino acids long, has an equal distribution of all four nucleotides at every position, and the deletion rate is 0.5%, only 0.18% will be in-frame over their entire lengths and free of stop codons. Consequently, unless the ORF is very short, one may wish to preselect the individual cassettes for being in-frame and free of stop codons. This preselection strategy is most easily accomplished by encoding different protein affinity tags close to the 3′- and 5′-termini of the cassettes. Synthesizing mRNA-displayed proteins from each individual DNA cassette, and purifying these upon the basis of the presence of each of these tags, will enrich the resultant library in those sequences that have initiated before the 5′ tag, terminated after the 3′ tag, and do not contain stop codons. These are likely to be in-frame over their entire sequence. The full-length DNA library should then be constructed from these preselected cassettes using RT-PCR followed by restriction and ligation. Any reduction in diversity that results from the preselection process is regained by the combinatorial ligation of the amplified DNA cassettes during the assembly of the full-length library. The steps in a preselection strategy are shown in Figure 24.5.3, and a detailed description is given in Cho et al. (2000).

Step	Product	
Synthesis and denaturing PAGE purification of DNA cassette(s), which when ligated together will encode the complete library	ssDNA cassette(s)	
PCR amplification of DNA cassette(s)	ds DNA cassette(s)	
Transcription of DNA cassette(s)	mRNA cassette(s)	
Denaturing PAGE purification of RNA cassette(s)	Purified mRNA cassette(s)	
Synthesis and denaturing PAGE of DNA splint that anneals to and aligns 3′-end of RNA and 5′-end of DNA linker to encourage ligation	DNA splint	
Synthesis and denaturing PAGE of DNA linker that terminates in puromycin	DNA linker	
Kinase (5′-phosphorylation) of DNA linker	Kinased DNA linker	
Splinted ligation of RNA cassette(s) to DNA linker terminated in puromycin	mRNA display template(s) of cassette(s)	
Translation of mRNA display template(s) of cassettes and high salt incubation and/or incubation at – 20°C	Protein cassettes with both affinity tags displayed upon their stop codon-free and frameshift-free mRNA display templates	
	Misinitiated protein cassettes without an N-terminal affinity tag displayed upon partly untranslated mRNA display templates	
	Frameshifted protein cassettes without a C-terminal affinity tagdisplayed upon mRNA display templates with deletions	
	mRNA display templates with stop codons not displaying proteins and untranslated mRNA display templates	
	Free protein cassettes	
	Reticulocyte lysate mRNA	
	Reticulocyte lysate	

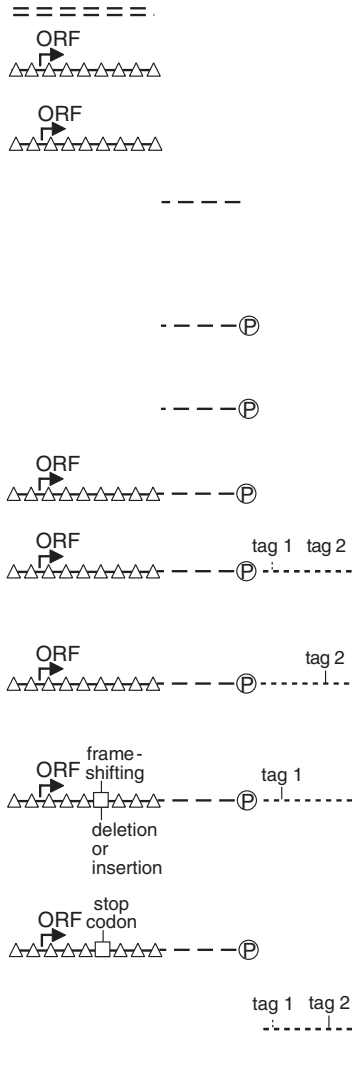


Figure 24.5.3 The steps that comprise the preselection process. A DNA cassette library that, when assembled into a full-length DNA library will encode a protein library, is enriched in those cassettes that are free of stop codons, insertions, and deletions. These cassettes are then used to construct the full-length library that is used for protein selection using mRNA display (*continues on next page*).

RNA polymerase promoter sequence

The library will need a transcription promoter at the 5′-end. This can be added or changed by PCR. The promoter sequences, TAATACGACTCACTATA and TTCTAATACGACTCACTATA, have both been successfully used. Transcription is most efficient if the RNA transcript starts with at least two guanines. To avoid pyrimidines (T or C) in the first few nucleotides of the transcript, it is common for the transcribed RNA sequence to commence with GGG.

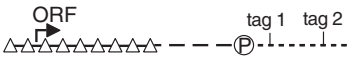
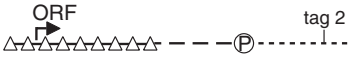
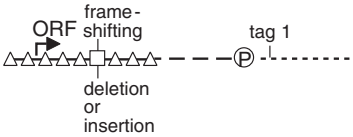
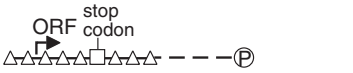
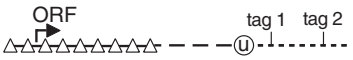
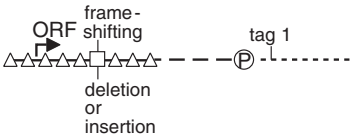
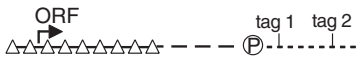
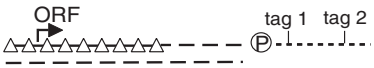
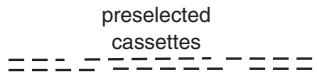
Step	Product	
Oligo(dT) cellulose purification	Protein cassettes with both affinity tags displayed upon their stop codon-free and frameshift-free mRNA display templates	
	Misinitiated protein cassettes without an N-terminal affinity tag displayed upon partly untranslated mRNA display templates	
	Frameshifted protein cassettes without a C-terminal affinity tag displayed upon mRNA display templates with deletions	
	mRNA display templates with stop codons not displaying proteins and untranslated mRNA display templates	
	Reticulocyte lysate mRNA	
N-terminal protein affinity tag (such as FLAG) purification (optionally repeated)	Protein cassettes with both affinity tags displayed upon their stop codon-free and frameshift-free mRNA display templates	
	Frameshifted protein cassettes without a C-terminal affinity tag displayed upon mRNA display templates with deletions	
C-terminal protein affinity tag (such as His_6) purification (optionally repeated)	Protein cassettes with both affinity tags displayed upon their stop codon-free and frameshift-free mRNA display templates	
Reverse transcription with the splint as primer	Protein cassettes with both affinity tags displayed upon their stop codon-free and frameshift-free and reverse transcribed mRNA display templates	
PCR amplification	dsDNA cassette(s) that are in-frame at both their 3′ and 5′-ends and free of stop codons	
Restriction, native PAGE purification, ligation, native PAGE purification, optionally repeated one or more times	Full-length DNA library encoding an open reading frame substantially free of stop codons, deletions, and insertions	

Figure 24.5.3 Continued.

Translation enhancer sequence

The library will need a translation enhancer before the initiating methionine codon; the truncated 5′-UTR from the Tobacco Mosaic Virus sequence (ACAATTACTATTTA-CAATTACA) has been used successfully.

Initiating methionine

The initiating methionine (ATG) immediately follows the translation enhancer sequence.

N-terminal constant ORF sequence

It is extremely helpful to have amino acid sequences within the protein that can act as affinity tags. These are invaluable when purifying the displayed proteins. If two different affinity tags are used and these are located close to each of the termini of the expressed

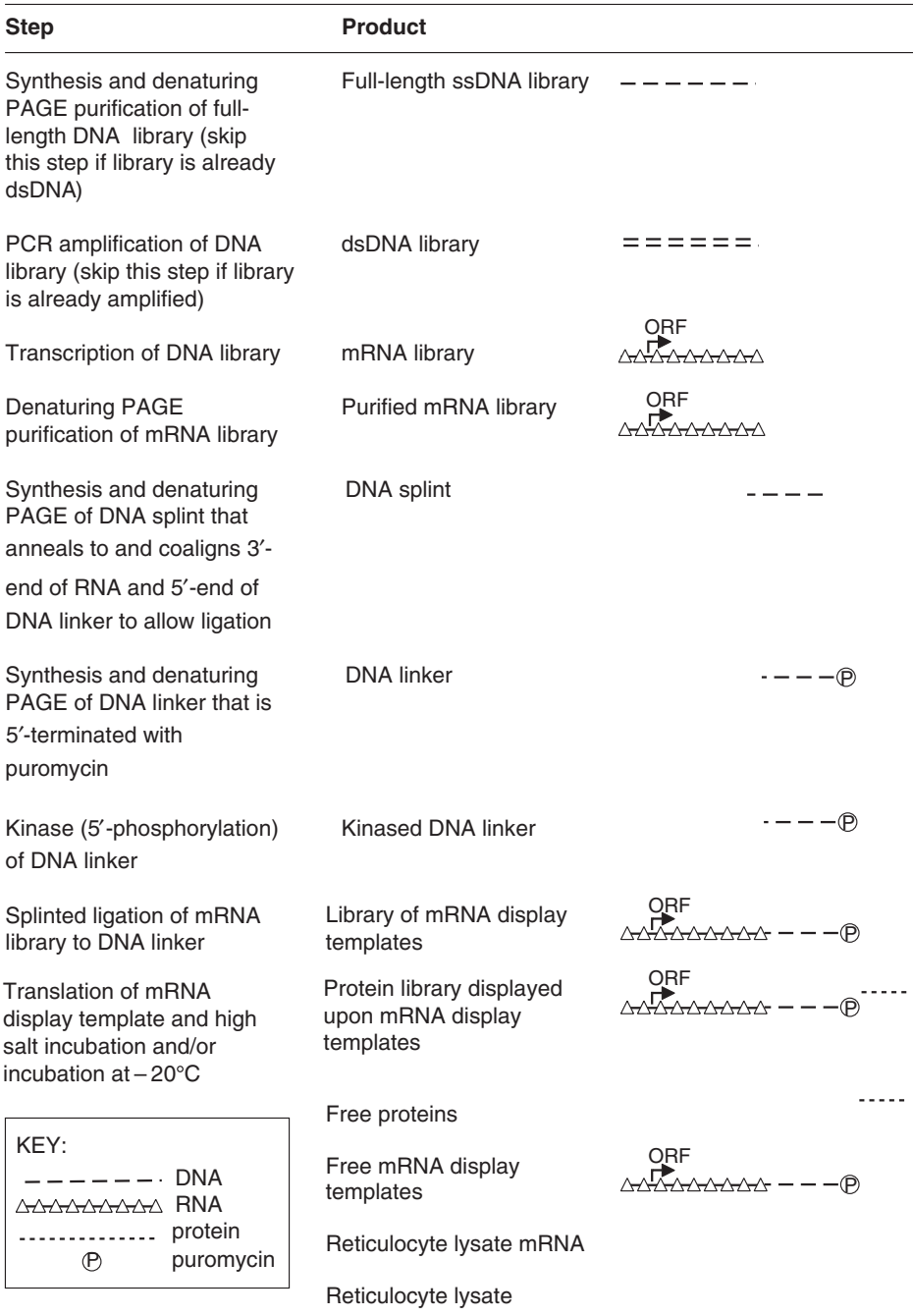
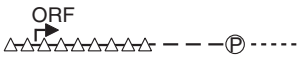
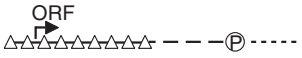
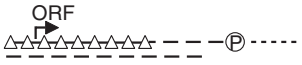
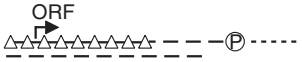
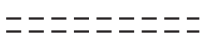

Step
Product
Synthesis and denaturing PAGE purification of full-length DNA library (skip this step if library is already dsDNA)
Full-length ssDNA library
PCR amplification of DNA library (skip this step if library is already amplified)
dsDNA library
Transcription of DNA library
mRNA library
ORF
Denaturing PAGE purification of mRNA library
Purified mRNA library
ORF
Synthesis and denaturing PAGE of DNA splint that anneals to and coaligns 3′-end of RNA and 5′-end of DNA linker to allow ligation
DNA splint
Synthesis and denaturing PAGE of DNA linker that is 5′-terminated with puromycin
DNA linker
Ⓟ
Kinase (5′-phosphorylation) of DNA linker
Kinased DNA linker
Ⓟ
Splinted ligation of mRNA library to DNA linker
Library of mRNA display templates
ORF
Ⓟ
Translation of mRNA display template and high salt incubation and/or incubation at – 20°C
Protein library displayed upon mRNA display templates
ORF
Ⓟ
Free proteins
Free mRNA display templates
ORF
Ⓟ
Reticulocyte lysate mRNA
Reticulocyte lysate
KEY:
DNA
RNA
protein
Ⓟ
puromycin

Figure 24.5.4 The steps that comprise a single round of selection and amplification in a protein selection using mRNA display (*continues on next page*).

protein, then they may be used to ensure that the expressed protein, mRNA-displayed or not, is full-length and in-frame at both ends. This double purification may optionally be performed when the DNA library is still at the individual cassette stage in order to increase the proportion of library members that are full-length, in-frame, and do not contain stop codons (see Fig. 24.5.3). FLAG and His_6-tag sequences are obvious choices.

Some constant amino acid sequence is likely to result from the ligation junctions used to construct libraries from synthetic DNA with long random regions; the identity of these

Step	Product	
Oligo(dT) cellulose purification	Protein library displayed upon mRNA display templates	
	Free mRNA display templates	
	Reticulocyte lysate mRNA	
Protein affinity tag (such as FLAG or His_6) purification	Protein library displayed upon mRNA display templates	
Reverse transcription with the splint as primer	Protein library displayed upon reverse transcribed mRNA display templates	
Selection step	Selected members of the protein library displayed upon reverse transcribed mRNA display templates	
PCR amplification of selected fraction of library	Input dsDNA library for the next cycle of selection and amplification	
Repeat procedure from the start until the proportion of the library detected in the selected fraction has peaked or reached a plateau, at which point the library should be sequenced and individual members should be assayed for activity.	Individual functional proteins that pass the selection test with which they have been challenged	

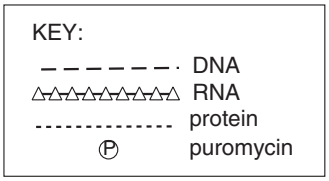


Figure 24.5.4 Continued.

amino acids and their frame can be adjusted in order to avoid "inappropriate" amino acids such as several consecutive hydrophobic residues or a proline. What is considered to be inappropriate will depend upon what the library is to be used for. Regardless of how the library is to be constructed, having different restriction sites encoded within the 3′ and 5′ ends of the open reading frame will allow for the changing of one or other of the protein terminal constant sequences if design considerations change, or should reengineering be required for troubleshooting.

Long stretches of uridines in the RNA sequence should be avoided since these may anneal to the poly(dA) sequence of the puromycin-terminated linker oligonucleotide. This will interfere with the ligation used to construct the mRNA display template. Also, the double-stranded RNA-DNA, which can result from the self-annealing of the resulting

mRNA display template, will act as a substrate for the RNase H, which is present in reticulocyte lysate, resulting in degradation of the mRNA display template.

C-terminal constant ORF sequence

All of the considerations described with regard to the N-terminal constant ORF sequence also apply to the C-terminal constant ORF sequence. There are also additional considerations that are specific to the C-terminal sequence.

In the synthesis of the mRNA display template (see Fig. 24.5.4), the 3′ terminus of the mRNA encoding the ORF is ligated to a short DNA oligonucleotide, which is itself 3′-terminated with puromycin (the linker). The 3′ terminus of the mRNA and the 5′ terminus of the DNA linker are coannealed to a short DNA oligonucleotide (the splint), which is complementary to both of them and presents the junction to be ligated as a nicked double-stranded nucleic acid. Consequently, the secondary structure of the splint, the mRNA, and the linker should be checked for self-structure likely to interfere with the assembly of the splinted nicked double-stranded complex. This can most easily be done using a computer algorithm such as MFOLD (*http://www.ibc.wustl.edu/~zuker/rna/form1.cgi*).

The mRNA-displayed protein is attached to the mRNA via its C terminus. It seems appropriate, therefore, to make the last few amino acids at the C terminus "structureless," i.e., a stretch of glycines and serines. Incorporating extra methionines into the constant sequence will increase the signal resulting from the incorporation of ^{35}S-methionine into the protein. Extra methionines are best placed in the C-terminal constant region downstream of the C-terminal protein affinity tag. Should they result in misinitiation, then the resultant proteins will not contain either of the protein affinity tags and will not copurify with the mRNA-displayed full-length proteins. Placing out-of-frame stop codons close to the C terminus, either before or after the tag, in both the +1 and −1 frames will prevent those members of the library that are out-of-frame at the C terminus from forming mRNA-displayed proteins. This is especially useful in the context of a preselection (see Fig. 24.5.3). Incorporating a protein kinase (phosphorylation) site to allow ^{32}P-labeling of the protein may assist in assaying the free proteins.

Statistical appearance of different amino acids within the random region

Most DNA libraries designed for protein selection encode a wide range of amino acids in their random regions. Using a mixture of all four nucleotides at each of the three positions in the library codons will ensure that all 20 amino acids have some probability of appearing in every position of the resulting protein sequence. During the library design process it is helpful to consider the average composition of amino acids that will result from the chosen nucleotide distribution, the consequent average proportions of hydrophobic and charged amino acids, and whether these proportions are suitable for the library and its intended target. Additionally, it is useful to consider the frequency with which certain individual amino acids will appear in the random region. Cysteines can coordinate transition metal ions or form disulfide bonds, histidines can accept or donate protons or coordinate transition metal ions, and prolines may disrupt secondary structure. Other specific amino acids may be suitable for interacting with intended substrates.

Frequency of stop codon appearance in random libraries

Using a mixture of all four nucleotides at each of the three positions in the library codons in the DNA encoding the protein library will also introduce stop codons. This will reduce the proportion of expressed protein that is displayed because stop codons will cause the ribosome to release the mRNA before the terminating puromycin is able to react with the nascent peptide. By altering the proportions of the nucleotides in the DNA synthesis mixtures, the frequency of stop codons can be reduced, although this will also influence

the proportions of other amino acids in the library. Alternatively, the DNA cassettes used to construct the library can be synthesized from mixtures of nucleotides chosen only with regard to the average amino acid composition they encode. The resultant cassettes can then be "preselected" as described above in order to enrich the resultant library with those that do not contain stop codons. As shown in Fig. 24.5.3, if this procedure is done using two different affinity tags at the different termini of the ORF, then the resultant library will also be enriched in cassettes without deletions. A nucleotide distribution encoding a target amino acid composition can be iteratively approached using computer algorithms that are available on the Internet (e.g., *http://gaiberg.wi.mit.edu/cgi-bin/CombinatorialCodons*; Wolf and Kim, 1999).

Codon usage

Statistical studies of sequenced genomes have shown that, for the majority of amino acids for which more than one codon exists, certain codons appear more frequently than others. The more frequently used codons tend to end in G or C, which may relate to the extra stability that results from having three hydrogen bonds in the wobble position of the tRNA-mRNA complex. Consequently, it may be helpful to design the library so that G and C are the only nucleotides in the third position of each codon. The symmetry of the genetic code is such that the composition of the wobble position has very little effect upon the composition of the resultant protein, so this approach need not affect the amino acid composition of the protein library it encodes. However, this strategy will amplify the effect that frameshifts will have upon the resultant proteins.

Periodicity and stop codon avoidance

Some mixtures of nucleotides may result in the total omission of stop codons, $(VNN)_n$ for example (where V is a mixture of A, G, and C) does not encode any stop codons. Unfortunately, such approaches always result in the loss of some of the 20 amino acids; $(VNN)_n$ also does not encode Cys, Phe, Trp, and Tyr for example. Mixing different such codons together can give a DNA library that encodes all 20 amino acids but no stop codons. This approach, however, necessarily introduces an element of design into the library, since the statistically different codons must be placed at specified points in the sequence, most usually in a periodic fashion. Periodicity can also result in an increased tendency for protein structural units to be encoded; for example, alternate hydrophobic and hydrophilic amino acids will encourage the formation of β sheets, while alternate pairs of hydrophobic and hydrophilic amino acids will encourage the formation of α helices. Alternatively, there are nonperiodic nucleotide distributions that reduce the occurrence of stop codons to ~1%. For an example of this approach see LaBean and Kauffmann (1993).

Mutagenesis

Mutagenic procedures such as mutagenic PCR (Cadwell and Joyce, 1992) or DNA shuffling (Stemmer, 1994) may be used to generate a diverse DNA library from a less diverse DNA library or a single DNA sequence (a minimum of two homologous sequences is required for DNA shuffling). Mutagenic procedures may be used to generate the initial DNA library for an mRNA display protein selection, or to increase the diversity at any stage between cycles of selection and amplification during an mRNA display protein selection. In general, in vitro selection proceeds by the gradual loss of diversity as functional sequences are preferentially amplified and nonfunctional sequences are lost. Consequently, increasing the diversity of the library at a stage after the first selection step may appear to be a retrogressive step; however, this is not necessarily the case. Protein libraries that are generated by stochastic means, such as those generated from DNA made on a DNA synthesizer using mixtures of nucleotide phosphoramidites, sample protein sequence space extremely sparsely. For example, a member of a library of 10^{13} proteins

each of which is 72 amino acids long, with each amino acid being equally likely to appear at each position, will, on average, have a sequence with 26 differences ("mutations") from the next most similar member of the library. If a solution to a particular problem is chanced upon using such a library, it is highly unlikely to be the optimal solution. The solution in question may be a small number of mutations away from many other superior solutions, but the initial library is extremely unlikely to have contained any of the sequences in question because the sampling was so sparse. Once a selection strategy has given one or many such nonoptimal solutions, one or more mutagenic steps will enable the exploration of the local sequence space around such solutions and, after subsequent selection, is likely to yield improved solutions closely related to one or more of the originally selected sequences.

Specific directions for the preparation of a DNA library for an mRNA display selection vary greatly depending upon the source of the DNA, the selection target, and the precise assembly strategy chosen. See *UNITS 24.2, 24.3, & 24.4* for further details. A more detailed discussion of both preselection and mRNA display library construction strategy is given in Cho et al. (2000). A generalized library construction strategy is also shown in Fig. 24.5.5. Once the DNA library has been synthesized, but before the selection has commenced, it is important to sequence some of the individual library members to ensure that the library sequence is as intended, and that the proportion of error-free sequences is appropriate for the selection strategy envisaged.

Selection

In vitro selection strategies, such as mRNA display, offer a generalized method for the discovery of functional molecules, only if the molecules in question can be enriched upon the basis of their function and subsequently directly amplified. Enrichment upon the basis of function is termed selection. The appropriate design of the selection step is absolutely crucial to the success of the project as a whole. Most in vitro selection experiments have been performed upon libraries of nucleic acids rather than proteins (Wilson and Szostak, 1999), although some protein selection experiments have been successfully performed using ribosome display (Jermutus et al., 1998) and most recently mRNA display (A.D. Keefe and J.W. Szostak, pers. commun.). Phage display (Smith and Petrenko, 1997) and the 2-hybrid system (Fields and Song, 1989; Colas et al., 1996) are similar in vivo techniques that have also been successfully used for the selection of functional peptides and proteins.

In vitro selections are divided into two main categories, (1) selections for aptamers (i.e., specific binding to a chosen target) and (2) selections for catalysts (i.e., enzymes). This is not an appropriate place for an exhaustive overview of the various approaches that have been used to discover new aptamers and catalysts, but some general points can be made.

Aptamer selections

Aptamer (specific binder) selections are in general undertaken by incubating the library with the immobilized target molecule. The target molecule immobilization is by way of covalent attachment to a solid matrix such as agarose, usually through a spacer molecule. Many immobilized target molecules are commercially available. After incubation, the flowthrough is drained away, the immobilized target molecules are washed several times, and then an elution fraction is collected by incubating the matrix and the immobilized target molecules with an elution buffer that contains the dissolved target molecule. Those library members that are contained in the elution fraction are amplified, and the process is repeated until functional molecules dominate the library. At this stage, the functional molecules are identified by cloning and sequencing. It is important to realize that in the early rounds of selection, the vast majority of the library members contained in the elution

fraction are nonspecific binders or nonbinders. Consequently, several rounds of selection and amplification will be required before the functional (specific binding) sequences dominate the library.

The composition of the selection binding and selection elution buffers is likely to influence the aptamers that are ultimately discovered using this system. It may be important to use a buffer that promotes protein folding, but discourages aggregation. The use of high concentrations of cosmotropic compounds such as ammonium sulfate will promote folding, while the use of nonionic detergents such as Triton X-100 will discourage aggregation. The oxidation potential of the buffer should also be considered. The inclusion of a reducing agent such as DTT is likely to lead to the selection of protein aptamers active under reducing conditions, while the inclusion of oxidizing agents such as glutathione disulfide is likely to lead to the selection of protein aptamers active under oxidizing conditions. It is important to ensure that the binding and elution buffers are as similar as possible. Changes in ionic strength and/or pH between these buffers will increase the proportion of nonspecific binders in the elution fraction, possibly to such an extent that the specific binders will never dominate the library and consequently will never be identified. Obviously the binding and elution buffers cannot be identical since the elution buffer contains the target molecule. In order to make the selection binding and

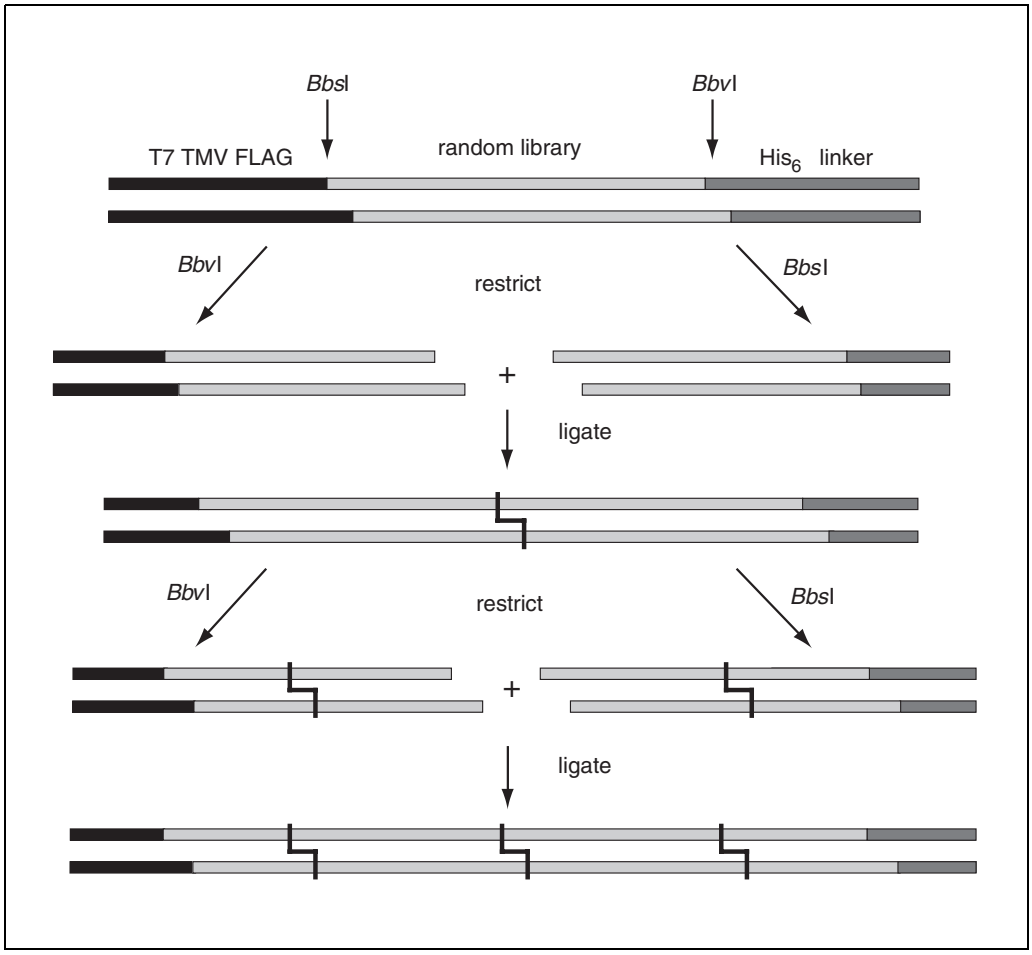


Figure 24.5.5 Assembly of a full-length mRNA display template library from DNA cassettes that result from the RT-PCR amplification of pre-selected mRNA display cassette templates. In this example, the cassettes are divided into 2 aliquots that are restricted with either *Bbv*I or *Bbs*I, subsequent ligation with T4 DNA ligase gives a new cassette in which the DNA between the restriction sites doubles in length while the flanking regions remain the same. The doubling of the length of this region may be repeated any number of times by repeating the restriction and ligation process.

selection elution buffers as similar as possible, it may be desirable to balance the effect of the target molecule upon the elution buffer by adding a similar molecule to the binding buffer. If the target molecule interacts with one of the buffer components, such as a nucleotide with magnesium, it may be desirable to add back an extra amount of this component to maintain the free concentration of the component in question at the concentration of that in the binding buffer. It is also possible to collect the elution fraction by disrupting the binding with denaturant or extremes of pH, although the background is likely to be higher.

Catalytic (enzyme) selections

Catalytic (enzyme) selections are a little more complex in a conceptual sense—although carefully designed, they can have lower intrinsic background rates than aptamer selections, and consequently be quicker and easier to perform in the laboratory. Enzyme selections must be performed in such a manner that those sequences which catalyze a reaction are separated from those that do not. The most obvious way to achieve this separation is to arrange the selection so that library members that catalyze the desired reaction covalently attach themselves to the substrate. If the substrate is in turn covalently attached to a tag (such as biotin), then the attachment of the tag to library members that catalyze the reaction can be used as a basis for the separation of these library members from those that do not catalyze the reaction (such as by binding to immobilized streptavidin). Alternatively the substrate may be immobilized before it is incubated with the library. Since both of these approaches effectively turn the catalysis selection into a binding selection, there will still be a background rate of isolation of sequences that do not catalyze the desired reaction. Consequently it may still be necessary to perform several rounds of selection and amplification before functional sequences dominate the library. Similar catalytic selection strategies can be envisaged in which all of the library members are immobilized, and those that successfully catalyze the desired reaction cut themselves free.

It should be noted that since the successfully selected library members are required to modify themselves in some respect, they are not acting as catalysts in the true sense of the word. However, molecules selected using such a procedure are usually easily reengineered to give true catalysts by detaching the active site part of the selected construct from the substrate part. One consequence of this limitation is that it is difficult to select for catalysts that act faster than the rate with which they can be manipulated in the laboratory, and it is not possible to select for catalysts with high turnover rates at all. Strategies in which the library member is encapsulated along with several substrate molecules may lead to systems in which the selective pressure is directly for the turnover rate.

Selection controls

The importance of selection controls cannot be emphasized strongly enough. mRNA display selection protocols in which functional library members are enriched much less than ten-fold over nonfunctional library members are unlikely to lead to the isolation of functional library members in the laboratory. Biases are present in many steps of the mRNA display amplification protocol, especially translation and protein display efficiencies, and these can overwhelm the enrichment in functional members that results from the selection step. Suitable positive controls are molecules known to catalyze or bind to the intended substrate, and need not be proteins, although the best control will usually be a similar functional protein displayed upon its reverse-transcribed mRNA display template.

Nomenclature

mRNA-displayed proteins are referred to by a variety of names in the literature, such as "RNA-protein fusions" and "profusions."

PREPARATION AND PURIFICATION OF mRNA-DISPLAYED PROTEINS

BASIC PROTOCOL 1

This protocol describes the preparation of the mRNA display template from an appropriate DNA template, DNA splint, and DNA linker 3′-terminated with puromycin, the use of the mRNA display template to prepare mRNA-displayed proteins and their subsequent purification, and an example selection. The protocol steps are also shown in Figure 24.5.4. For additional details see Liu et al. (2000).

Materials

DNA library
1 M $MgCl_2$
100 mM nucleotide triphosphate solutions
10× transcription buffer (see recipe)
Deionized, ultrafiltered water
10 U/µl T7 RNA polymerase
Solid EDTA
Urea
0.5× TBE buffer (*APPENDIX 2*)
3 M NaCl
100% and 70% ethanol
100 mM EDTA
Puromycin-terminated DNA linker
100 mM ATP
T4 polynucleotide kinase buffer
T4 polynucleotide kinase
10× T4 DNA ligase buffer
10 U/µl T4 DNA ligase
3 M potassium acetate solution, pH 5.3
Rabbit reticulocyte lysate translation kit (e.g., Red Nova Lysate kit, Novagen)
  Control RNA
  12.5× methionine-free translation mix
  2.5 M potassium chloride
  25 mM magnesium acetate
  Nuclease-free water
  Rabbit reticulocyte lysate
^{35}S-methionine

Electroeluter (VWR or Schleicher & Schuell)
Denaturing PAGE gel (*UNIT 2.12*)
Gel filtration columns (Pharmacia)

Additional reagents and equipment for preparative denaturing PAGE purification (*UNIT 2.12*), determining nucleic acid concentration by spectrometry (*APPENDIX 3D*), synthesis of oligonucleotides (*UNIT 2.11*), and SDS-PAGE in Tris-tricine buffer systems (*UNIT 10.2A*)

Transcribe DNA

1. Make up a 1-ml transcription reaction on ice as follows. Add the T7 RNA polymerase last.

   DNA library (add volume sufficient for 5 to 50 nM final concentration)
   35 µl 1 M $MgCl_2$
   50 µl each 100 mM nucleotide triphosphate (final 5 mM each NTP)
   100 µl 10× transcription buffer (final 1×)
   Up to 980 µl deionized, ultrafiltered water
   20 µl 10 U/µl T7 RNA polymerase (final 200 U/ml).

   Incubate the transcription reaction for 3 to 16 hr at 37°C. Halt the reaction by cooling on ice, or by adding solid EDTA to a final concentration of 50 mM.

   The size of the transcription reaction can be adjusted to give an appropriate amount of RNA, but care should be taken to ensure that the diversity of the DNA used is several times larger than the diversity of the displayed proteins that will ultimately result. The effect of varying the concentration of $MgCl_2$ should be explored in pilot transcriptions.

Purify RNA

2. Purify resultant RNA using denaturing PAGE (*UNIT 2.12*). Add solid urea to the transcription reaction to give a final concentration of 5 M and solid EDTA to give a final concentration of 50 mM, heat for 2 min at 90°C, and load onto a denaturing PAGE gel.

3. After the gel has been run, visualize by UV-shadowing and excise the band containing the purified RNA. Extract the RNA into 300 mM NaCl by passive elution or into 0.5× TBE buffer in an electroeluter according to the manufacturer's instructions.

4. Precipitate the RNA by adding 3 M NaCl (final concentration 300 mM) and 2.5 volumes of 100% ethanol. Cool for 20 min at −80°C or overnight at −20°C.

5. Centrifuge for 10 min at 12,000 × *g*, 4°C. Decant the supernatant, wash the pellet with 70% ethanol, dry under reduced pressure, bring up to 0.5 ml with deionized, ultrafiltered water, and measure the concentration by UV-visible spectroscopy at 260 nm.

   Further instructions may be found in UNIT 2.12.

   This purification step separates truncated RNA molecules and PCR primers from the full-length RNA transcripts. It is important to remove the PCR primers from the transcribed RNA since they will inhibit the formation of the mRNA displayed proteins in the translation step.

Synthesize linker

6. Synthesize the linker, a DNA oligonucleotide 3′-terminated in puromycin, that is ~30 nucleotides long and "unstructured" (e.g., according to one of the following examples; see *UNIT 2.11* for oligonucleotide synthesis):

   Example a. AAAAAAAAAAAAAAAAAAAAAAAAAACCP.

   Poly(dA) is the most obvious choice.

   Example b. AAAAAAAAAAAAAAAAAAA999ACCP.

   In example b, "9" is phosphoramidite spacer 9 (Glen Research) and "P" is puromycin, derived from CPG-puromycin (Glen Research). This linker may give a higher yield of displayed proteins.

Linkers much longer or shorter than 30 nucleotides will give greatly reduced yields of displayed proteins, or none at all.

The puromycin-terminated DNA oligonucleotide is gel purified by denaturing PAGE (UNIT 2.12), extracted from the gel, and precipitated as described earlier. Dissolve the DNA linker in deionized water and measure the concentration using UV-visible spectroscopy at 260 nm.

Each of the spacer 9 units result in the incorporation of a triethylene glycol phosphate ester; this adds extra flexibility to the region of the template close to the puromycin and may result in a higher proportion of the resultant mRNA display templates displaying protein.

7. Kinase (5′-phosphorylate) the DNA linker using polynucleotide kinase by making up the following 1-ml kinase reaction mixture:

   300 µl 100 µM DNA linker (final 30 µM)
   10 µl 100 mM ATP (final 1 mM)
   100 µl 10× T4 polynucleotide kinase buffer (final 1×)
   490 µl water
   100 µl 10 U/µl T4 polynucleotide kinase (final 200 U/ml).

8. Incubate the reaction mixture for 2 hr at 37°C, add 200 µl of 100 mM EDTA, heat for 5 min at 90°C, and desalt on a gel-filtration column.

   It is important to heat-denature the polynucleotide kinase to prevent it from acting in the subsequent ligation reaction. The size of the kinase reaction should be adjusted to give an appropriate amount of 5′-phosphorylated DNA linker.

Synthesize splint

9. Synthesize the splint, a DNA oligonucleotide with a sequence (reading from the 5′ end) of ≥10 nucleotides complementary to the 3′ end of the RNA library and ≥10 nucleotides complementary to the 5′ end of the linker, usually T_{10} (see *UNIT 2.11* for oligonucleotide synthesis methods). Purify by denaturing PAGE (*UNIT 2.12*).

10. Extract DNA from gel as in step 3 and precipitate as in step 4.

11. Dissolve the purified splint in deionized water and measure the concentration using UV-visible spectroscopy at 260 nm (see step 5).

Prepare mRNA display template

12. Ligate the linker and RNA template with T4 DNA ligase in the presence of the splint to give the mRNA display template. Set up the following 1-ml ligation reaction:

    100 µl 100 µM 5′-phosphorylated DNA linker (final 10 µM)
    100 µl 100µM RNA library (final 10 µM)
    100 µl 100 µM splint (final 10 µM)
    580 µl water.

13. Heat this mixture for 2 min at 95°C, then add 100 µl of 10× T4 DNA ligase buffer (final 1×).

14. Vortex the resultant mixture and cool on ice for 10 minutes, allow to warm to room temperature, then add 20 µl of 2000 U/µl T4 DNA ligase (final 40 U/ml).

15. Incubate the reaction for 20 min at room temperature. Add 150 µl of 100 mM EDTA and 500 mg of solid urea, and heat for 5 min at 90°C.

Table 24.5.1 Translation Reactions for mRNA Display Proteins

Reagent	Final concentration	A	B	C	D	E	F
Control RNA	—	1 µl	0 µl	0 µl	0 µl	0 µl	0 µl
5 µM mRNA display template	2/4/800 nM	0 µl	0 µl	0 µl	1 µl	2 µl	4 µl
5 µM unligated RNA	400 nM	0 µl	0 µl	2 µl	0 µl	0 µl	0 µl
12.5× Met-free translation mix	1×	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
8.6 µM labeled methionine	0.69 µM	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
2.5 M KCl	100 mM	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
25 mM magnesium acetate	500 µM	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl
Water	—	8.5 µl	9.5 µl	7.5 µl	8.5 µl	7.5 µl	5.5 µl
2.5× rabbit reticulocyte lysate[a]	1×	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
Total		25 µl	25 µl	25 µl	25 µl	25 µl	25 µl

[a]Ensure that the rabbit reticulocyte lysate is added last.

16. Gel purify ligated mRNA display template by denaturing PAGE (*UNIT 2.12*), extract from gel (see step 3), and precipitate as in step 4, except use 3 M potassium acetate, pH 5.3, in place of 3 M sodium chloride.

17. Dissolve the purified ligated mRNA display template in deionized water and measure the concentration by UV-visible spectroscopy at 260 nm.

If the template is <500 nucleotides long, it should be possible to resolve the ligated and unligated RNA on the PAGE gel, which will give some idea of the yield of the ligation reaction. Otherwise, the unresolved bands will have to be co-excised from the gel and optionally further purified using oligo(dT) cellulose as described below. It is important to perform this gel purification even if it is not possible to resolve the ligated and unligated RNA, since the presence of the splint in the translation reaction will greatly reduce the yield of displayed proteins, and RNase H in the reticulocyte lysate will cause degradation of the mRNA display template if it is annealed to the splint. It should be noted that this splinted RNA-DNA ligation is far less efficient than the ligation of sticky-ended pieces of DNA.

Translate mRNA display template and prepare mRNA displayed proteins

Before the mRNA display template is used for large-scale translation, a small-scale translation should be attempted alongside various control translations to aid the identification of the band on the protein gel that corresponds to the mRNA-displayed proteins.

18. Set up the translation reactions in Table 24.5.1 on ice, adding the rabbit reticulocyte lysate last.

19. Incubate for 1 hr at 30°C, then add 1.7 µl of 1 M $MgCl_2$ and 7.8 µl of 2.5 M KCl to each of the reactions and allow them to stand for 5 min at room temperature.

The reaction mixtures may be optionally stored for up to 1 week at −20°C at this point.

20. Analyze the different translations using Tris-tricine SDS-PAGE as described in *UNIT 10.2A*, Alternate Protocol 1.

The SDS-PAGE analysis should show a number of bands in lane A, which is the control RNA supplied by the manufacturer; this is the positive control and demonstrates that the translation reaction was set up correctly. Lane B is the no-template control, and may show no bands or may show a band corresponding to tRNA charged with methionine; in either case it should show no bands with mobilities equal to those assigned to the free protein and to the displayed protein. Lane C should show a band of high mobility that can be

assigned to the free protein. Lanes D, E, and F should also show bands that can be assigned to the free protein, but also bands of much lower mobility that can be assigned to the mRNA-displayed protein. If the density of the band assigned to the displayed protein in F is of equal or lesser density to the equivalent band in E, then the mRNA display template is likely to be of high quality.

For better proof that the band assigned to the mRNA displayed proteins is done so correctly, add splint (final 1 μM) and $MgCl_2$ (final 10 mM) to an aliquot of the translation mixture before the salt incubation, and incubate for 30 min at 37°C. RNase H within the lysate will cause the RNA part of the mRNA-displayed proteins to be digested and leave the protein displayed upon the DNA linker alone, consequently the original displayed protein band will disappear and a new band will appear of intermediate mobility between the mRNA displayed protein and the free protein.

21. Once the displayed proteins have been observed by SDS-PAGE, optimize the magnesium acetate and potassium chloride concentrations in the translation reaction. Perform a succession of translations in parallel with added magnesium acetate concentrations ranging from 0.5 mM to 2 mM and added potassium chloride concentrations ranging from 50 mM to 200 mM.

    The relative proportions of the mRNA display templates that end up displaying proteins will be readily apparent when the samples are run out on a gel together, and the optimal concentrations of both magnesium acetate and potassium chloride can be chosen.

    If a preselection procedure is being used to synthesize the full-length mRNA display template library, the translation magnesium acetate and potassium chloride concentrations will have to be optimized separately for each cassette used in the preselection protocol, and then again for the full-length library. Despite the fact that the 3′-terminal region of each of the cassettes and the full-length library are the same, the optimal magnesium acetate and potassium chloride concentrations for the formation of mRNA-displayed proteins are likely to be different.

22. Prepare a 1-ml translation reaction on ice as follows, adding the reticulocyte lysate last.

    80 μl 5 μM mRNA display template (final 400 nM)
    80 μl 12.5× methionine-free translation mix (final 1×)
    20 μl 8.6 μM [^{35}S]methionine (final 0.17 μM)
    2.5 M KCl (as optimized)
    0.5 μl magnesium acetate (as optimized)
    Water to 600 μl
    400 μl 2.5× rabbit reticulocyte lysate (final 1×)
    Total, 1000 μl.

23. Incubate for 1 hr at 30°C, then add 65 μl of 1 M $MgCl_2$ and 235 μl of 2.5 M KCl to each of the above reactions and allow them to stand for 5 min at room temperature.

    The translation reaction mixtures may be optionally stored for up to a week at −20°C at this point.

    One may wish to decrease the concentration of mRNA display template if there is concern about sequence-dependent bias in translation and protein display efficiencies affecting the distribution of different sequences in the library. As the concentration of the mRNA display template is reduced, the proportion of this template that ends up displaying protein increases, with a concomitant increase in the fidelity with which the mRNA library sequence distribution is represented in mRNA-displayed protein sequence distribution. This is an advisable precaution at all stages in which the library is of relatively low diversity.

BASIC PROTOCOL 2

PURIFICATION AND REVERSE TRANSCRIPTION OF THE mRNA-DISPLAYED PROTEINS

It is extremely advisable to pilot each of the following protocol steps before attempting the large-scale treatment of translation reaction mixture containing mRNA-displayed proteins.

In order to separate the mRNA display templates that display proteins from those that do not, it is necessary to use a purification step upon the basis of a protein affinity tag. In this protocol the His_6 tag is used, although other protein affinity tags may be utilized.

Materials

Oligo(dT) cellulose (Amersham Pharmacia Biotech)
Oligo(dT) binding buffer (see recipe)
1.3-ml translation reaction mRNA displayed proteins (see Basic Protocol 1)
Oligo(dT) wash buffer (see recipe)
Ni-NTA agarose (Qiagen)
Ni-NTA binding buffer (see recipe)
2-Mercaptoethanol
Ni-NTA wash buffer 1 (see recipe)
Ni-NTA wash buffer 2 (see recipe)
Ni-NTA elution buffer (see recipe)
10 mg/ml salmon sperm DNA (Life Technologies)
1 mg/ml BSA
200 µM DNA splint
5× Superscript II reverse transcriptase buffer (NEB)
0.1 M DTT
30 µl (each) 25 mM deoxynucleotide triphosphates (final 0.5 mM)
200 U/ml Superscript II reverse transcriptase (NEB)
25 mM deoxynucleotide triphosphate solutions
ATP-aptamer selection binding buffer (see recipe)
ATP-aptamer selection elution buffer (see recipe)

Chromatography columns (Bio-Rad)
Gel filtration columns (e.g., NAP-5, Amersham Pharmacia Biotech)

For additional reagents and equipment for preparative denaturing PAGE purification (*UNIT 2.12*) and SDS-PAGE in Tris-tricine buffer systems (*UNIT 10.2A*)

Purify mRNA displayed proteins

1. Wash 20 mg of oligo(dT) cellulose repeatedly with deionized water in the chromatography column within which it will be used. Resuspend the cellulose several times and apply positive pressure to force it to drain rapidly. Finally, wash once with oligo(dT) binding buffer.

   Oligo(dT) cellulose contains fine particulate matter that can drastically reduce the flow rate of aqueous solutions. These fine particles can also pass through the frit during use of the chromatography column, which will result in the loss of mRNA-displayed proteins. This step forces the finest particles through the frit, and is especially important with the use of larger amounts of oligo(dT) cellulose.

2. Dilute the 1.3-ml translation reaction containing the mRNA-displayed proteins with added KCl and $MgCl_2$ (from Basic Protocol 1) into 8.7 ml of oligo(dT) binding buffer and incubate with the washed oligo(dT) cellulose for 15 min at 4°C with rotation.

   Retain an aliquot of the undiluted translation reaction for SDS-PAGE and scintillation counting analyses.

3. Allow the diluted translation reaction mixture and the oligo(dT) cellulose to pass through a chromatography column so that the oligo(dT) cellulose is retained on the frit, and retain the flowthrough.
4. Wash three times with 1 ml oligo(dT) binding buffer.
5. Wash once with 1 ml oligo(dT) wash buffer.
6. Elute three times with 0.5 ml deionized water.
7. Analyze the undiluted translation reaction mixture, the flowthrough, and all of the washes and elutions using Tris-tricine SDS-PAGE as described in *UNIT 10.2A*, Alternate Protocol 1, and by scintillation counting.

   The volume of the oligo(dT) eluate may be reduced by lyophilization by up to a factor of 5.

   The oligo(dT) cellulose purification step anneals the poly(dA) region of the mRNA display template to immobilized oligo(dT) cellulose. Consequently, mRNA display templates not displaying protein and other mRNA molecules present in the lysate will co-purify with the displayed proteins. Long stretches of adenines in the RNA region of the mRNA display template should be avoided since they will also anneal to the oligo(dT) and present a substrate for RNase H which is present in the reticulocyte lysate; this will cause mRNA display template degradation.

   The oligo(dT) cellulose purification step also presents a quick approximate method for the absolute measurement of the concentration of mRNA displayed proteins in the translation reaction mixture. The scintillation counter readings give the proportion of ^{35}S-methionine that is contained in the oligo(dT) eluates by counting equal proportions of the whole translation mixture and the oligo(dT) eluate and dividing one by the other. The ratio of the intensities of the bands corresponding to mRNA-displayed proteins in these two samples on the SDS-PAGE gel gives the yield of the oligo(dT) purification.

8. Calculate the concentration of mRNA displayed proteins in the translation reaction mixture with the following equation:

   $$[\text{mRNA-displayed proteins}] = Y^{-1} \times C \times [\text{methionine}] \times N^{-1}$$

   where Y is the yield of the oligo(dT) cellulose purification determined by SDS-PAGE; C is the number of counts in the combined oligo(dT) elution fractions divided by the number of counts in an equal proportion of the translation reaction mixture determined by scintillation counting; [methionine] is the total concentration of hot and cold methionine in the translation reaction mixture before the high salt incubation; and N is the average number of methionines in a single displayed protein.

   This calculation assumes that the initiating methionine is still present on the protein and that the concentration of methionine in the reticulocyte lysate is known (~5 μM before addition to the translation reaction); this last error can be reduced by adding a known amount of cold methionine to the translation reaction mixture.

   A potentially more accurate method for the direct measurement of the concentration of mRNA displayed proteins is to construct the mRNA display template using a mixture of DNA linker that has been kinased (5′-phosphorylated) with labeled ATP as well as the cold kinased (5′-phosphorylated) linker. This labeled template can then be translated in the presence of only cold methionine. It is generally not possible to observe the difference in mobility on a SDS-PAGE gel between the mRNA display template displaying protein and the mRNA display template alone. The addition of a DNA oligonucleotide, complementary to a region of the RNA part of the mRNA display template close to but not right at the 3′ end of the RNA, to the translation reaction mixture, as well as magnesium chloride to 10 mM, will cause the RNA part of the mRNA-displayed proteins to be digested away by RNase H, which contaminates reticulocyte lysate, leaving proteins displayed upon the ^{32}P-labeled DNA linker only. These may easily be resolved from the DNA linker not displaying protein using SDS-PAGE. The ratio between these two bands gives a more direct measurement of

the proportion of mRNA display template that displays protein, and using this the concentration of mRNA-displayed proteins in the translation reaction mixture may easily be calculated. The proportion of mRNA display template that ends up displaying protein can vary from <1% to 40% depending upon the sequence, the myc epitope sequence is at the upper end of this range.

Ni-NTA purification

The Ni-NTA purification is upon the basis of the His_6 tag and is only appropriate if this is present in the library sequence (see also *UNIT 10.11*).

9. Wash 100 µl of Ni-NTA agarose three times with 1 ml deionized water.
10. Mix 0.5 ml of the oligo(dT) eluate with 2× Ni-NTA binding buffer, vortex to dissolve, add 0.7 µl of 2-mercaptoethanol, incubate with the washed Ni-NTA agarose for 1 hr at 4°C with rotation.

    The 2× Ni-NTA binding buffer is the solid residue obtained by evaporation to dryness of 1× Ni-NTA binding buffer.

11. Allow the Ni-NTA binding buffer and the Ni-NTA agarose to pass through a chromatography column so that the Ni-NTA agarose is retained on the frit, retain the flowthrough.
12. Perform the following washes on the chromatography column:
    a. Wash two times with 500 µl Ni-NTA wash buffer 1.
    b. Wash once with 500 µl of a 4:1 solution of Ni-NTA wash buffer 1:Ni-NTA wash buffer 2.
    c. Wash once with 500 µl of a 3:2 solution of Ni-NTA wash buffer 1:Ni-NTA wash buffer 2.
    d. Wash once with 500 µl of a 2:3 solution of Ni-NTA wash buffer 1:Ni-NTA wash buffer 2.
    e. Wash once with 500 µl of a 1:4 solution of Ni-NTA wash buffer 1:Ni-NTA wash buffer 2.
    f. Wash once with 500 µl Ni-NTA wash buffer 2.
    g. Wash two times with 500 µl of a 19:1 solution of Ni-NTA wash buffer 2:Ni-NTA elution buffer.
    h. Elute for 30 min at 4°C with rotation two times with 250 µl Ni-NTA elution buffer.

    EDTA should be added to the eluate to give 5 mM to bind to eluted Ni^{2+}.

13. Analyze the starting material, the flowthrough, and all washes and elutions using Tris-tricine SDS-PAGE as described in *UNIT 10.2A*, Alternate Protocol 1, and by scintillation counting.

    The volume of the eluate may be reduced by lyophilization by up to a factor of 5.

    If the mRNA-displayed proteins are prone to aggregation, then it may be necessary to maintain denaturing conditions throughout the Ni-NTA agarose purification process by the addition of urea or guanidinium hydrochloride to the wash and elution buffers in addition to that which is in the binding buffer.

    If it is not desired to completely denature the mRNA-displayed proteins, the denaturant can be omitted from all buffers including the binding buffer; using such a native Ni-NTA agarose purification procedure is likely to result in decreased yields compared to the denaturing Ni-NTA agarose purification procedure described above.

    The Ni-NTA agarose purification is upon the basis of the His_6 tag. Alternatively, other protein-affinity tags may be encoded within the protein sequence and used as a basis for

purification, such as the FLAG tag (see Support Protocol 1). The Ni-NTA purification will separate the mRNA-displayed proteins from the mRNA display templates not displaying proteins and other mRNA molecules that were not purified away in the oligo(dT) purification. The Ni-NTA agarose eluate will, however, contain contaminating free library protein if this is present in the input mixture; in this protocol this is removed in the preceding oligo(dT) purification. If it is desired to purify the free library protein, then it is best to purify the translation mixture initially upon the basis of a FLAG tag, with the Ni-NTA agarose purification subsequent to this. Additionally, a denaturing His_6 tag purification may be used to purify selected mRNA-displayed proteins away from the selection binding buffer if more than one selection step is to be used between amplification steps and the denaturing and renaturing of the mRNA-displayed proteins is desired.

Strong chelating agents such as EDTA, EGTA, and DTT must be avoided in Ni-NTA binding and wash buffers since they will compete with the immobilized NTA for complexation of the Ni^{2+}, and may elute it from the agarose.

Purify on gel filtration column

14. On a NAP-5 gel filtration column, exchange the elution buffer into water by allowing 10 ml of deionized water to flow through the gel-filtration column.

15. Add 100 µl of 10 mg/ml salmon sperm DNA and 10 µl of 1 mg/ml BSA to 890 µl of deionized water, vortex, and allow this to flow through the gel filtration column.

16. Allow 10 ml of deionized water flow through the gel filtration column.

17. Allow 0.5 ml of sample to flow through the gel filtration column.

18. Add 1 ml of deionized water to the column and collect the 1-ml eluate issued from bottom of column.

19. Analyze the starting material and the elution fraction using Tris-tricine SDS-PAGE as described in *UNIT 10.2A*, Alternate Protocol 1 and by scintillation counting.

Imidazole does not inhibit reverse transcription, so this buffer exchange is optional, it may be possible to reverse transcribe the mRNA display templates by diluting the Ni-NTA eluate directly into the reverse transcription reaction mixture. Reverse transcription may not proceed if denaturants are present in the reaction mixture.

Reverse transcribe mRNA-displayed proteins

20. Set aside a small sample of the mRNA-displayed proteins that are not reverse-transcribed for use in the no-RT control PCR amplification.

21. Make up the following reverse transcription reaction mixture on ice. Mix mRNA displayed proteins and DNA splint (functions as the RT primer) together first before adding RT buffer, and add reverse transcriptase last:

    900 µl mRNA-displayed proteins
    15 µl 200 µM DNA splint (final 2 µM)
    300 µl 5× reverse transcription buffer (final 1×)
    150 µl 100 mM DTT (final 10 mM)
    30 µl (each) 25 mM deoxynucleotide triphosphates (final 0.5 mM)
    5 µl water
    10 µl 200 U/µl Superscript II reverse transcriptase (final 1333 U/ml)
    Total, 1500 µl.

    Incubate the reverse transcription reaction for 50 min at 42°C.

22. Analyze the starting material and the product of the reverse transcription using Tris-tricine SDS-PAGE as described in *UNIT 10.2A*, Alternate Protocol 1, and by scintillation counting.

The volume of the eluate may be reduced by lyophilization by up to a factor of 5. The reverse-transcribed mRNA-displayed proteins have greater mobility on the SDS-PAGE gel compared to those that have not been reverse transcribed. This difference in mobility provides a simple method for the accurate assay of the proportion of the mRNA displayed proteins which have been reverse transcribed. However, this change in mobility may only be observed if the cDNA-RNA association is preserved during the treatment of the sample during gel loading, which may not be the case if it is heated too strongly (much above 90°C).

It is common practice in reverse-transcription reactions to heat denature the primer and RNA template before the addition of the reverse transcriptase; this may influence the conformation of the mRNA displayed proteins, and depending on the project may not be advisable. Mixing the primer and the mRNA display template together under low-salt conditions, before the addition of the buffer, should promote their association.

The use of mRNA displayed proteins in selection experiments may yield functional RNA sequences unless the mRNA display template is reverse transcribed before the selection step. This will also reduce the likelihood that the mRNA display template will disrupt the structure of the protein that it displays. Free proteins originally selected using mRNA display may need to be incubated under reverse transcription conditions in order to achieve their active conformations.

Purify RT products

23. Exchange the buffer into selection buffer on a NAP-5 gel filtration column according to the manufacturer's instructions.
24. Allow 10 ml of selection binding buffer to flow through the gel filtration column.
25. Add 100 µl of 10 mg/ml salmon sperm DNA and 10 µl of 1 mg/ml BSA to 890 µl of selection binding buffer, vortex, and allow this to flow through the gel filtration column.
26. Allow 10 ml of selection binding buffer to flow through column and 0.5 ml of sample to flow through column.
27. Add 1 ml of selection binding buffer to the top of the gel filtration column and collect the 1-ml eluate issued from bottom of column.
28. Analyze the starting material and the elution fraction using Tris-tricine SDS-PAGE as described in *UNIT 10.2A*, Alternate Protocol 1, and by scintillation counting.

The selection binding buffer used here is specific to the selection being performed.

Alternatively, a protein-folding step may accompany the buffer exchange into selection buffer. In this approach a denaturant such as guanidinium hydrochloride or urea is added directly to the mRNA-displayed proteins after reverse transcription, and this is dialyzed away over several hours into selection buffer. It is important to ensure that the denaturing conditions are not so denaturing that the association between the cDNA and the mRNA display template is broken; this may be assayed using SDS-PAGE.

BASIC PROTOCOL 3

SELECTION AND AMPLIFICATION OF THE mRNA-DISPLAYED PROTEINS

Selection protocols are highly project-dependent. The following protocol was successfully used to select ATP-binding proteins from a random sequence library and is included as an example. Cycles of selection and amplification, as described in this protocol, should be repeated until the proportion of the resultant mRNA-displayed proteins in the selected fraction is no longer increasing—typically, 8 to 12 cycles are required. At this point the selected library sequences should be determined by cloning and sequencing (see Chapters

1 and 7), and individual clones should be assayed for activity under the selection conditions both as mRNA-displayed and free proteins.

Materials

ATP agarose (Sigma)
ATP-aptamer selection binding buffer (see recipe)
Purified mRNA-displayed proteins (see Basic Protocol 2)
ATP-aptamer selection elution buffer (see recipe)
100 mM EDTA (*APPENDIX 2*)
1 M NaOH (*APPENDIX 2*)
1 M HCl
10 mg/ml salmon sperm DNA
1 mg/ml BSA
100 µM 3′ primer (specific for cDNA library)
100 µM 5′ primer (specific for cDNA library)
25 mM (each) deoxynucleotide triphosphates
10× PCR buffer containing 15 mM $MgCl_2$ (Boehringer Mannheim)
5 U/µl *Taq* DNA polymerase (Boehringer Mannheim)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol
Chloroform
1-Butanol
3 M NaCl
100% ethanol
Gel filtration columns (e.g., NAP-25, Amersham Pharmacia Biotech)

Additional reagents and equipment for butanol extraction (*UNIT 2.1A*)

Select for ATP-binding proteins

1. Wash 10 mg of ATP-agarose three times with 1 ml of deionized water followed by two times with 1 ml of ATP-aptamer selection binding buffer.

2. Incubate 1 ml of the purified mRNA-displayed proteins, from Basic Protocol 2, with the washed ATP-agarose for 1 hr at 4°C with rotation; drain for flowthrough.

3. Wash six times with l000 µl ATP-aptamer selection binding buffer at 4°C; allow to stand 10 min between washes.

4. Elute six times with 250 µl ATP-aptamer selection elution buffer at 4°C; allow to stand 10 min between elutions.

5. Assay all fractions using scintillation counting.

If the proportion of mRNA-displayed proteins in the elution fraction is high (>5%), it may be helpful to perform more than one selection step between amplification steps. In the case of an aptamer selection, this will necessitate the purification of the selected mRNA-displayed proteins away from the elution buffer. This purification can be performed while preserving native conditions, and often directly in the selection buffer, upon the basis of the FLAG tag, as described in Support Protocol 1. Alternatively, this purification can be performed with a denaturing and renaturing step, optionally upon the basis of the His_6 tag under denaturing conditions, as described above.

Purify selected cDNA sequences that encode selected mRNA-displayed proteins

6. To 1.5 ml of eluted mRNA displayed proteins, add 200 µl of 100 mM EDTA and 200 µl of 1 M NaOH, heat for 10 min at 90°C, cool on ice, and add 200 µl of 1 M HCl.

7. Exchange the buffer into deionized water on a NAP-25 gel filtration column according to the manufacturer's instructions.

8. Allow 25 ml of deionized water to flow through the column.
9. Add 200 µl of 10 mg/ml salmon sperm DNA and 20 µl of 1 mg/ml BSA to 1780 µl of deionized water, vortex, and allow this to flow through column.
10. Wash with 25 ml of deionized water and allow water to flow through the gel filtration column.
11. Measure the sample volume and pass through column, then add a volume of deionized water to the column such that the total volume added to the column is 2.5 ml.
12. Add 3.5 ml of deionized water to the top of the gel filtration column and collect the 3.5-ml eluate issued from the bottom of column.

With the exception of the hydrolysis step, this buffer exchange procedure may be optionally repeated after the volume of the sample has been reduced to ≤2.5 ml by evaporation under reduced pressure.

Amplify selected sequences by PCR

13. Amplify selected sequences by PCR (see also *UNIT 15.1*). Make up a PCR reaction mixture on ice as follows:

3500 µl selected cDNA library (from step 12)
100 µl 100 µM 3′ primer (final 2 µM)
100 µl 100 µM 5′ primer (final 2 µM)
40 µl (each) 25 mM deoxynucleotide triphosphates (final 0.2 mM)
500 µl 10× PCR buffer containing 15 mM $MgCl_2$ (final 1×)
735 µl water
25 µl 5 U/µl *Taq* DNA polymerase
Total, 5000 µl.

The number of cycles, temperatures, and durations of the incubation periods within each cycle need to be determined for the specific library being used (UNIT 15.1). The PCR amplification of DNA libraries should be piloted, and care should be exercised not to over-PCR amplify DNA libraries since they will not reanneal once denatured. If PCR is continued upon a denatured DNA library, rare sequences will be amplified to a greater extent than common sequences, which will reduce the enrichment factor of the selected functional sequences.

14. Perform a no-RT control (set aside in Basic Protocol 2, step 20) alongside this PCR reaction.

In this control a small amount of the mRNA display template that has not been reverse transcribed is used in place of the selected cDNA library. This should not give any observable product after an equivalent amount of amplification. If it does, then either the buffers are contaminated or the purification of the mRNA-displayed proteins is not stringent enough. In either case the problem must be addressed or the selection is unlikely to give the desired result. It is also often useful to perform an additional no-template control in which no template, reverse transcribed or otherwise, is added. If this gives observable product after an equivalent amount of amplification, then this is usually a sign of contaminated reagents.

Mutations will be introduced into the DNA library during PCR amplification. The mutagenic rate can be decreased by using a high-fidelity DNA polymerase such as Pfu DNA polymerase (e.g., Stratagene). The mutagenic rate can be increased by using the mutagenic PCR protocol described in Support Protocol 2. Mutagenic procedures such as mutagenic PCR may be used to increase library diversity by exploring parts of sequence space proximate to the starting sequence(s).

Purify double-stranded PCR product

15. Add 1:1 molar equivalents of 100 mM EDTA to chelate the Mg^{2+}.
16. Vortex the PCR reaction mixture with an equal volume of 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol, centrifuge for 1 min at 10,000 × *g*, room temperature, remove and retain upper aqueous phase.
17. Re-extract aqueous phase with an equal volume of chloroform three times; centrifuge to clear on each occasion, remove and discard lower organic phase after each centrifugation.
18. 1-Butanol extract the aqueous phase to 20% of the initial volume at minimum (*UNIT 2.1A*, Support Protocol 2), remove and discard the upper 1-butanol phase.

    Perform extraction in a polypropylene tube, as butanol will damage polystyrene.

19. Add 3 M NaCl to final 300 mM (include the salt that originates from the PCR buffer in calculating concentration) and 2.5 volumes of 100% ethanol.
20. Cool for 20 min at −80°C or overnight at −20°C. Centrifuge for 10 min at 12,000 × *g*, 4°C. Decant and discard the supernatant.
21. Centrifuge the pellet for 1 min at 12,000 × *g*, remove remaining supernatant with a plastic pipet tip, make up in 30 mM NaCl, and measure the concentration by agarose gel (more explicit instructions may be found in *UNIT 2.12*).

    The dsDNA library will not re-anneal if denatured, so care should be taken not to expose it to low-salt or high temperature conditions.

22. Transcribe the DNA into RNA, (see Basic Protocol 1) and repeat the entire procedure (see Basic Protocols 1, 2, and 3).

FLAG TAG PURIFICATION

SUPPORT PROTOCOL 1

The FLAG tag purification may optionally be used in place of or in addition to the His_6 tag purification in the purification of mRNA-displayed proteins. The FLAG tag purification is usually performed in addition to the His_6 tag purification during the preselection of individual cassettes during the library construction process. In this instance, the FLAG tag and His_6 tag are placed at opposite protein termini; purification upon the basis of the presence of both tags ensures that the protein is full-length and in-frame at both termini. This in turn ensures that the mRNA cassette that encodes the protein is free of insertions, deletions, and stop codons, and is suitable for the preparation of the full-length library by restriction and ligation of the resulting PCR-amplified cDNA sequences. In addition, FLAG tag purification may be used to purify selected mRNA-displayed proteins away from the selection binding buffer if more than one selection step is to be used between amplification steps and the denaturing and renaturing of the mRNA-displayed proteins is not desired.

Alternatively, FLAG tag purification may be used to purify free proteins away from reticulocyte lysate.

The FLAG purification is upon the basis of the FLAG tag sequence (DYKDDDDK) and is only appropriate if this is present in the library (see Strategic Planning).

Additional Materials *(also see Basic Protocol 1)*

Anti-FLAG M2 agarose (Sigma)
FLAG clean buffer (see recipe)
FLAG binding buffer (see recipe)
FLAG peptide (Sigma)

1. Wash 100 µl of anti-FLAG M2 agarose three times with 1 ml of FLAG clean buffer, and then three times with 1 ml of FLAG binding buffer.
2. Exchange sample buffer into FLAG binding buffer according to the directions presented in Basic Protocol 1 for other buffer exchanges.

   Optionally, dilute the sample buffer into the FLAG binding buffer or attempt purification directly from selection elution buffer.
3. Place 1 ml of the sample containing the mRNA-displayed proteins onto the washed anti-FLAG agarose and incubate for 1 hr at 4°C with rotation, drain, and retain flowthrough.
4. Wash the anti-FLAG agarose three times with 1 ml of FLAG binding buffer.
5. Elute from the anti-FLAG agarose two times with 0.5 ml FLAG binding buffer containing 10 µM of the FLAG peptide, 30 min for each elution at 4°C with rotation.

   If the FLAG tag purification is to be followed by a denaturing His_6 tag purification, then the elution fraction may be added directly to the 2× Ni-NTA binding buffer.

SUPPORT PROTOCOL 2

MUTAGENIC PCR

Mutagenic PCR may be used to increase the diversity of the DNA library that encodes the protein library. Mutagenic PCR may be used to generate the initial library, or to explore parts of sequence space proximate to the starting sequence(s). A broader discussion of the use of mutagenic PCR may be found in Cadwell and Joyce (1992).

Before the entire mutagenic protocol is enacted, it is important to pilot the PCR conditions to ensure that primer dimers are not taking over, and that the amplification per cycle is at least 1.7 to 1.8. The optimum PCR amplification conditions may be different from non-mutagenic PCR amplification performed upon the same library. One may wish to redesign the primers, since the part of the template sequence they anneal to will not be mutagenized.

Additional Materials *(also see Basic Protocol 3)*

2.5 M KCl
100 mM $MnCl_2$ solution
100 mM Tris·Cl, pH 8.3 (*APPENDIX 2*)
100 µl PCR tubes (Sarstedt)

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 15.1*)

1. Make up the following PCR reaction mixture on ice:

   100 µl 100 µM 3′ primer (final 2 µM)
   100 µl 100 µM 5′ primer (final 2 µM)
   60 µl (each) 25 mM dCTP and dTTP (final 1 mM)
   12 µl (each) 25 mM dATP and dGTP (final 0.2 mM)
   30 µl 2.5 M KCl (final 50 mM)
   10.5 µl 1 M $MgCl_2$ (final 7 mM)
   7.5 µl 100 mM $MnCl_2$ (final 0.5 mM)
   150 µl 100 mM Tris·Cl, pH 8.3 (final 10 mM)
   943 µl water
   15 µl 5U/µl *Taq* DNA polymerase
   Total, 1500 µl.

2. Pipet 16 90-µl aliquots of PCR reaction mixture into 100-µl PCR tubes and label them 1 to 16.

   These may be stored for up to a few hours at 4°C.

3. Add the DNA library or sequence to tube 1 to give 10 nM, make up to 100 µl with PCR reaction mix.

4. Perform 4 cycles of PCR amplification. During the final extension incubation, place the next-numbered tube alongside the current one in the PCR block. Before the final extension is complete but ensuring that the next-numbered tube is at the extension temperature, transfer 10 µl of PCR reaction mixture. Retain the amplified PCR reaction mixture at 4°C.

5. Repeat step 4 fourteen times. Every four transfers, analyze the PCR reaction using agarose gel electrophoresis (*UNIT 15.1*), quantitate the bands in successive PCR amplifications, and adjust the transfer volume in order to maintain the concentration of amplified DNA at a constant level.

   It is important not to over-PCR the DNA. If PCR amplification ceases before a concentration of 100 nM is reached, then the initial DNA concentration should be reduced accordingly.

   If the initial DNA was of one or a small number of known sequences, then it is possible to directly measure the average mutagenic rate by sequencing some of the individual library members from the final mutagenic PCR amplification sample. Assuming that the mutagenic rate is constant throughout the procedure allows for the direct control of the extent of mutagenesis by choosing one, or a mixture of more than one, of the successive mutagenic PCR amplification mixtures to serve as the source of the new DNA library. This sample may then be further amplified with PCR, optionally with further mutation. It is expected that the mutagenic rate will be about 0.2% per nucleotide per transfer (ten-fold amplification).

REAGENTS AND SOLUTIONS

The water used to make the following buffers should be deionized, ultrafiltered and subsequently tested for the absence of RNase by incubation with ^{32}P-labeled RNA and denaturing PAGE analysis. All buffers should be analyzed similarly. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

ATP-aptamer selection binding buffer

39.0 mg $MgCl_2$ (mol. wt. 95.2; 4.1 mM final)
2.92 g KCl (mol. wt. 74.6; 392 mM final)
476 mg HEPES (mol. wt. 238; 20 mM final)
3.07 mg glutathione (mol. wt. 307; 2 mM final)
3.06 mg glutathione disulfide (mol. wt. 612; 1 mM final)
3.72 mg EDTA·2Na$^+$ (mol. wt. 372; 100 µM final)
250 µl Triton X-100 (0.25% final)
Bring up to 100 ml with water
Store at −20°C

Deoxygenate the buffer before the addition of the glutathione by bubbling an oxygen-free grade of an inert gas such as argon or nitrogen through it, and adjust the pH to 7.4.

ATP-aptamer selection elution buffer

285 mg ATP·2Na$^+$ (mol. wt. 569; 5 mM final)
84.7 mg $MgCl_2$ (mol. wt. 95.2; 8.9 mM final)
2.92 g KCl (mol. wt. 74.6; 392 mM)
476 mg HEPES (mol. wt. 238; 20 mM final)

continued

3.07 mg glutathione (mol. wt. 307; 2 mM final)
3.06 mg glutathione disulfide (mol. wt. 612; 1 mM final)
3.72 mg EDTA·$2Na^+$ (mol. wt. 372; 100 µM final)
0.25 g Triton X-100 (0.25% w/v final)
Bring up to 100 ml with water
Store at −20°C

Deoxygenate the buffer before the addition of the glutathione by bubbling an oxygen-free grade of an inert gas such as argon or nitrogen through it, and adjust the pH to 7.4.

FLAG binding buffer

877 mg NaCl (mol. wt. 58.4; 150 mM final)
1.19 g 50 mM HEPES (mol. wt. 238; 50 mM final)
0.25 g Triton X-100 (0.25% w/v final)
Adjust the pH to 7.4 with NaOH/HCl
Bring up to 100 ml with water
Store at −20°C

FLAG clean buffer

751 mg glycine (mol. wt. 75.1; 100 mM final)
0.25 g Triton X-100 (0.25% w/v final)
Bring up to 100 ml with water
Store at −20°C
Adjust pH to 3.5 with NaOH/HCl.

Ni-NTA binding buffer

57.4 g guanidine hydrochloride (mol. wt. 95.5; 6 M final)
2.93 g NaCl (mol. wt. 58.4; 500 mM final)
1.42 g Na_2HPO_4 (mol. wt. 142; 100 mM final)
121 mg Tris(hydroxymethyl)aminomethane (mol. wt. 121; 10 mM final)
0.25 g Triton X-100 (0.25% w/v final)
701 µl 2-mercaptoethanol (mol. wt. 78.1; 10 mM final)
Adjust the pH to 8.0 with NaOH/HCl
Bring up to 100 ml with water
Store at −20°C

In order to prepare 2× Ni-NTA binding buffer, the 1× Ni-NTA binding buffer should be evaporated to dryness under reduced pressure. Upon using the resultant 2× Ni-NTA binding buffer, the 2-mercaptoethanol will have to be added again.

Ni-NTA elution buffer

2.93 g NaCl (mol. wt. 58.4; 500 mM final)
121 mg 10 mM Tris(hydroxymethyl)aminomethane (mol. wt. 121; 10 mM final)
0.25 g Triton X-100 (0.25% w/v final)
1.70 g imidazole (mol. wt. 68.1; 250 mM final)
701 µl 2-mercaptoethanol (mol. wt. 78.1; 10 mM final)
Adjust pH to 8.0 with NaOH/HCl
Bring up to 100 ml with water
Store at −20°C

Ni-NTA wash buffer 1

48.1 g urea (mol. wt. 60.1; 8 M final)
2.93 g NaCl (mol. wt. 58.4; 500 mM final)
1.20 g NaH_2PO_4 (mol. wt. 120; 100 mM final)
121 mg Tris(hydroxymethyl)aminomethane (mol. wt. 121; 10 mM final)
0.25 g Triton X-100 (0.25% w/v final)

continued

701 µl 2-mercaptoethanol (mol. wt. 78.1; 10 mM final)
Adjust the pH to 6.3 with NaOH/HCl
Bring up to 100 ml with water
Store at −20°C

Ni-NTA wash buffer 2

2.93 g NaCl (mol. wt. 58.4; 500 mM)
121 mg Tris(hydroxymethyl)aminomethane (mol. wt. 121; 10 mM final)
0.25 g Triton X-100 (0.25% w/v final)
701 µl 2-mercaptoethanol (mol. wt. 78.1; 10 mM final)
Adjust the pH to 8.0 with NaOH/HCl
Bring up to 100 ml with water
Store at −20°C

Oligo(dT) binding buffer

7.46 g KCl (mol. wt. 74.6; 1 M final)
1.21 g Tris(hydroxymethyl)aminomethane (mol. wt. 121; 100 mM final)
372 mg disodium EDTA (mol. wt. 372; 10 mM final)
0.25 g Triton X-100 (0.25% w/v final)
Adjust the pH to 8.0 with NaOH/HCl
Bring up to 100 ml with water
Store at −20°C

Oligo(dT) wash buffer

746 mg KCl (mol. wt. 74.6; 100 mM final)
121 mg Tris(hydroxymethyl)aminomethane (mol. wt. 121; 10 mM final)
0.25 g Triton X-100 (0.25% w/v final)
Adjust the pH to 8.0 with NaOH/HCl
Bring up to 100 ml with water
Store at −20°C

Transcription buffer, 10×

255 mg spermidine trihydrochloride (mol. wt. 255; 10 mM final)
4.84 g Tris(hydroxymethyl)aminomethane (mol. wt. 121; 400 mM final)
770 mg DTT (mol. wt. 154; 50 mM final)
0.1 g Triton X-100 (0.1% w/v final)
Adjust the pH to 8.0 with NaOH/HCl
Bring up to 100 ml with water
Store at −20°C

COMMENTARY

Background Information

In vitro selection experiments were first successfully performed upon nucleic acid libraries, for reviews see Szostak and Ellington (1993), Gold et al. (1993), and Joyce (1993). Nucleic acids are the only molecular systems that are capable of being replicated directly in vitro and which also can contain more than trivial amounts of amplifiable information. Nucleic acid selections have the advantage that the target functional entity, and the information encoding the functional entity, are the same. Within nucleic acid selections, large libraries (i.e., up to 10^{17} different molecules) are subjected to successive cycles of selection and amplification until functional sequences dominate the library, at which point they may be identified by cloning and sequencing. The idea of extending the in vitro selection approach to proteins was an obvious one; what was not obvious, however, was how to extract the sequence information from selected proteins in order to permit their amplification and ultimately their identification.

One way to extract the sequence information from selected proteins is to covalently attach each of them to the mRNA sequence that encodes it (Roberts and Szostak, 1997; Roberts,

1999; Roberts and Ja, 1999). In this manner the function (phenotype) and amplifiable sequence information (genotype) are part of the same molecule. Selection may be performed upon the basis of the function of the protein, while the protein may be amplified upon the basis of the mRNA that encodes it and is attached to it. The problem was how to arrange things so that proteins may be attached to the mRNA that encodes them in parallel while mixed within one reaction mixture. The molecule that makes this possible is puromycin (Fig. 24.5.1). Puromycin is an antibiotic that functions by inhibiting translation. It sufficiently closely resembles a charged tRNA that it is able to enter the A-site of the ribosome and react with the activated C-terminal of the nascent peptide. Since this reaction results in the formation of a stable amide bond, rather than the hydrolyzable ester bond that connects the tRNA to the amino acid in an amino-acyl tRNA, translation is halted. If the puromycin is already attached to the mRNA that is being translated, a stable covalent linkage results between a protein and the mRNA that encodes it and the ribosome may be purified away.

In a conceptual sense, the most similar systems to mRNA display are phage display (Smith and Petrenko, 1997) and ribosome display (Jermutus et al., 1998). All three systems may be used to search nucleic acid libraries for the functional proteins or peptides they encode. In phage display, the protein library is encoded within the phage genome and is expressed upon the surface of the phage as a fusion with the phage coat protein. Phage may be selected upon the basis of the functionality of their surface proteins, and the protein may then be amplified by allowing the selected phage to replicate. The diversity of phage display selection experiments is limited to the numbers of phage that may reasonably be transformed or packaged, which is ~10^8 to 10^9. Recent advances have shown that it is possible to display libraries of proteins upon the surface of phage (Sche et al., 1999), but phage display has yet to be used to discover a new protein from an entirely random sequence library, as distinct from a library derived from a known folded protein. In ribosome display, paused ribosomes display the protein library in a ternary complex with the mRNA that encodes the displayed protein. In order to synthesize this ternary complex, an mRNA library is prepared without a stop codon. Upon translation of this template the ribosomes will pause at the end of the open reading frame. Because the ribosome is unable to release itself from the message, it displays both the nascent protein and the mRNA that encodes it. These constructs may be used for in vitro selection experiments upon the basis of the function of the displayed nascent protein. Selected proteins may then be amplified using RT-PCR amplification of the associated mRNA. The ribosome display constructs are relatively large, and selections can only be performed under conditions that preserve the ribosome-mRNA-nascent protein association. Also, since the ribosome display constructs also display a single-stranded mRNA, there is a possibility that functional RNA sequences may be selected in place of the desired functional proteins. This problem can be avoided by reverse transcribing the mRNA associated with the ribosome.

Critical Parameters

After designing and synthesizing the mRNA display library, it is important to have it sequenced in order to ascertain the proportion of the library which is error-free and appropriate for the selection. If the proportion of library members with insertions, deletions, or stop codons is too high, the library may have to be resynthesized with extra purification steps incorporated at the DNA cassette stage (pre-selection, see Fig. 24.5.3). Alternatively, considerable library quality improvement may result from the careful denaturing PAGE purification of small amounts of the DNA cassettes.

The various purification steps performed after the initial synthesis of the mRNA-displayed proteins should be individually optimized, with assays performed by SDS-PAGE to ascertain that the mRNA displayed proteins are still attached to full-length mRNA. Subsequent to this, a pilot ("round zero") purification should be performed in which the various optimized purification steps are applied sequentially to the same sample. Only upon the satisfactory completion of round zero should the large-scale translation reaction be made up for the first round of selection ("round one").

The various purification steps that form part of each cycle of the selection of mRNA displayed proteins must be assayed by SDS-PAGE in order to confirm that the mRNA display template has not become degraded at any stage in the process.

Both positive and negative controls need to be used to assay the selection step; this should then be optimized to discriminate between the two controls to the maximum possible reasonable extent. This maximal discrimination selection protocol should be adopted after round two

or three; at this stage the absolute yield of the selection step is no longer a concern owing to the high copy number of selected sequences that have passed through ≥1 amplification step.

Alongside the PCR amplification that follows each selection step, a no-RT control should be performed. In this control a small amount of the mRNA display template that has not been reverse-transcribed is used in place of the selected cDNA library. This should not give any observable product after an equivalent amount of amplification. If it does, then either the buffers are contaminated or the purification of the mRNA-displayed proteins is not stringent enough. A no-template control PCR amplification will distinguish between these two possibilities. In either case, the problem must be addressed or the selection is unlikely to give the desired result.

Troubleshooting

Problems that may be encountered with this procedure are detailed in Table 24.5.2.

Anticipated Results

The results of a selection largely depend upon how many of the initial library members can perform the task for which they are being selected and how well they can perform it under the selected conditions. Ideally, the observed activity in each round of selection will exponentially rise to a high value and then plateau. Assuming that there are a relatively small number of members of the initial library with activity that causes them to be selected, it is likely that several rounds of selection and amplification will have to be performed before any significant increase in activity is observed.

Once the selection activity has peaked or reached a plateau, then the library members should be sequenced. If there were relatively large numbers of members of the initial library with activity that causes them to be selected, then the library at this stage may still be fairly diverse. Since the cycles of selection and amplification preferentially amplify the most active members, a high-diversity library at the end of the selection is likely to indicate a failed selection or a selection that is not yet finished. Successful selections are likely to yield one or a small number of families of very closely related protein sequences each of which has diverged from a single ancestral protein sequence owing to errors accumulated during the many cycles of PCR amplification to which they have been subjected. Assays of individual members of these selected families of sequences should yield mRNA-displayed proteins with the desired function; these are also likely to be functional as free proteins, unless the mRNA display template greatly interferes with the conformation of the displayed protein that it displays.

Early indications are that proteins selected using mRNA display may fold into multiple conformations, only some of which have the desired functionality. This behavior causes the proportion of the selected library observed to demonstrate the desired activity to rise by a factor of much less than might be expected during successive rounds of selection. The individual selected proteins behave similarly, whether mRNA displayed or not. Mutagenesis and reselection of such selected individual library members, or libraries of them, has given large families of related proteins with greatly improved characteristics in this respect (A.D. Keefe, G. Cho, and J.W. Szostak, pers. commun.).

It should always be borne in mind that selections will give a solution to the problem that is set; it is up to the experimenter to arrange the selection conditions sufficiently carefully to ensure that this solution is a consequence of the desired functionality.

The range of acceptable yields of various parts of the mRNA-displayed protein selection protocol are listed in Table 24.5.3. Observed yields falling at the lower end of these ranges may or may not be increased upon optimization.

Time Considerations

The construction of the library may take anywhere between 2 weeks and 2 months. Doing pilot preparative and purifying experiments on mRNA displayed protein may take ≥1 month. A single round of selection and amplification will take 2 to 4 days and the initial rounds of selection may take 1 to 2 months. Mutagenesis will take 1 to 2 weeks and subsequent rounds of selection and amplification will take 1 to 2 months. Sequencing and assays of selected proteins may take ≥1 month.

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Table 24.5.2 Troubleshooting Guide to Problems That May Be Encountered In Protein Selection Using mRNA-Displayed Proteins

Problem	Possible cause	Solution
Sequencing of initial library or library cassettes reveals many insertions and/or deletions	Synthetic DNA is of low quality	Repeat synthesis and/or careful denaturing PAGE purification to resolve *n* from *n*+1 and *n*−1 oligonucleotides
Sequencing of initial library or library cassettes reveals many insertions and/or deletions and/or stop codons	Synthetic DNA is of low quality and/or stop codons appear in random region as a consequence of library design	Perform "preselection" in which mRNA displayed proteins are synthesized at the cassette stage; these are purified upon the basis of the presence of both terminal tags, and the resulting cDNA is used to construct the full-length library (see Fig. 24.5.3)
mRNA-DNA ligation does not yield any/enough mRNA display template	3′-end of mRNA and/or splint have self-structure arising from internal complementarity	Redesign mRNA and/or splint sequences
	Puromycin-terminated linker was not sufficiently 5′-phosphorylated	Repeat 5′-phosphorylation, optionally with extra enzyme
	Too much salt in the ligation reaction mixture	Desalt mRNA, splint and linker
	RNA is degraded	Repeat transcription and gel purification
No mRNA-displayed proteins observed on gel	Ligation failed	See above
	mRNA display template is degraded	Repeat ligation
	No methionines present in library except initiating methionine which is degraded away in lysate	Redesign protein library with more methionines
Oligo(dT) cellulose purification low-yielding	Elution buffer not sufficiently denaturing	Further deionized water washes are needed to wash away residual salt
Ni-NTA purification low-yielding	His_6 tag not accessible	Use more denaturing conditions for the binding step or redesign library
	Product precipitates	Add denaturant to the wash and elution buffers
	EDTA, EGTA, DTT, or other chelating agents present in the binding buffer	Redesign protocol to exclude chelating agent
Library DNA observed in no-template control PCR amplification	PCR amplification components contaminated with library DNA	Determine which PCR amplification components are contaminated and replace them
Library DNA observed in no-RT control PCR amplification	Library DNA has not been purified away from mRNA displayed proteins, or mRNA displayed protein purification buffers are contaminated	Increase the stringency of the mRNA displayed protein purification protocol, or determine which mRNA displayed protein purification components are contaminated and replace them
Activity does not rise through selection	There are no functional sequences in library	Redesign or mutagenize library and reselect
	Selection step not designed appropriately	Test selection step with positive and negative controls, redesign to maximize distinction

continued

Table 24.5.2 Troubleshooting Guide to Problems That May Be Encountered In Protein Selection Using mRNA-Displayed Proteins, continued

Problem	Possible cause	Solution
	Biases in PCR, transcription, translation, or protein display overwhelming selection bias	Adjust conditions so that biases are reduced, especially in low-yielding steps; e.g., reduce mRNA display template concentration in translation
	Immobilized target not accessible to mRNA-displayed proteins	Repeat selection with different matrix and/or linker and/or target linkage point
No families observed in sequencing data at end of selection	Not enough cycles of selection and amplification performed	Continue with cycles of selection and amplification
	There are many sequences in the selected library that are active	Assay individual selected sequences
Selected sequences not active as mRNA-displayed proteins	Not enough cycles of selection and amplification performed	Continue with cycles of selection and amplification
	There are no functional sequences in library	Redesign or mutagenize library and reselect
Selected sequences active as mRNA-displayed proteins, but not as free proteins	Assay does not treat free proteins in exactly the same mannner as mRNA-displayed proteins	Repeat assay, treating free proteins in the same manner as mRNA-displayed proteins, for example include the reverse transcription step
	Selected mRNA-displayed proteins have mRNA-dependent conformations	Redesign or mutagenize library and reselect

Table 24.5.3 Results Obtained During mRNA-Displayed Protein Selection Procedure

Step	Range of acceptable yields
5′-phosphorylation of DNA linker	90%-100%
Splinted RNA-DNA ligation	20%-60%
Proportion of mRNA display template displaying protein	1%-40%
Oligo(dT) cellulose purification	30%-90%
Denaturing Ni-NTA purification	30%-90%
Anti-FLAG purification	50%-80%
Gel filtration chromatography (NAP column)	85%-100%
Reverse transcription	80%-100%
Proportion of mRNA displayed proteins in initial elution phase of aptamer selection	0.01%-1%
Proportion of mRNA displayed proteins in final elution phase of aptamer selection	3%-60%
Other statistics relating to mRNA display protein selections	
Number of rounds of selection until activity peaks or plateaus	8-12
Initial diversity of mRNA display library	10^{12}-10^{13}
Final diversity of mRNA display library	≥1-10^{4}

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Describes the optimization of the synthesis and purification of mRNA displayed proteins (RNA-protein fusions).

Cho et al., 2000. See above.

Describes the use of mRNA display and in vitro selection to construct various types of high quality library for use in mRNA display protein selections.

Internet Resources

http://gaiberg.wi.mit.edu/cgi-bin/CombinatorialCodons

Combinatorial Codons is an extremely useful tool for the design of protein libraries; it generates a nucleotide distribution that iteratively approaches an input amino acid distribution.

http://xanadu.mgh.harvard.edu/szostakweb/orf.html

This site is a database of exact oligonucleotide sequences that have been successfully used in the construction of random, patterned, and structure-based mRNA-displayed protein libraries.

http://paris.chem.yale.edu/extinct.html

The Biopolymer Calculator is a very useful general tool for molecular biology.

http://sun2.science.wayne.edu/%7Ejslsun2/servers/seqanal/

A nucleic acid secondary structure prediction algorithm is given by mfold.

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Directed Evolution of Proteins In Vitro Using Compartmentalization in Emulsions

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ABSTRACT

This unit describes a protocol for the directed evolution of proteins utilizing in vitro compartmentalization. This method uses a large number of independent in vitro transcription and translation (IVTT) reactions in water droplets suspended in an oil emulsion to enable selection of proteins that bind a target molecule. Protein variants that bind the target also bind to and allow recovery of the genes that encoded them. This protocol serves as a basis for carrying out selections in emulsions, and can potentially be modified to select for other functionalities, including catalysis. This selection method is advantageous compared to alternative selection protocols due to the ability to screen through very large-size libraries and the ability to express and screen or select for functions that would otherwise be toxic or inaccessible to in vivo selections and screens. *Curr. Protoc. Mol. Biol.* 87:24.6.1-24.6.12. © 2009 by John Wiley & Sons, Inc.

Keywords: directed evolution • in vitro compartmentalization • emulsion

BASIC PROTOCOL

Directed evolution (e.g., generation of a diverse initial library of molecules followed by selection for a desired function, typically carried out over iterative cycles or "rounds" of selection and amplification) of DNA, RNA, and proteins, can be carried out using many different methods. The goal of a directed evolution experiment is to identify one or few members of the starting library which perform the desired function at a high level. RNA and DNA selections can be performed in bulk solution by capturing molecules based on binding affinity or catalytic activity, and then directly amplifying the nucleic acids. Proteins, on the other hand, must be evolved under conditions in which genetic material is physically or spatially linked to the translated protein product. This can be carried out in several different ways. For example, a library can be cloned into a cell, and the expression of a particular, functional protein can lead to selection of both the cell and the cell's DNA. Similarly, phage display technologies link coding DNA to proteins displayed on the phage surface. Finally, mRNA display (Roberts and Szostak, 1997) uses the antibiotic puromycin to create a physical link between genetic material and protein during in vitro translation.

Most recently, methods have been developed for carrying out enzymatic reactions in the aqueous phase of an oil/water emulsion, including DNA replication (via PCR), transcription, and translation (Tawfik and Griffiths 1998; Ghadessy et al., 2001; Agresti et al., 2005; Levy et al., 2005). Transcription and translation can be coupled so that the entire pathway from DNA template (containing a protein-coding gene and the necessary regulatory sequences to initiate transcription and translation) to protein can be recapitulated ex vivo. These methods have served as the basis for selecting proteins from libraries, one of which is described in this unit. In addition, emulsion methods can be used for

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the selection of functional ribozymes (Agresti et al., 2005; Levy et al., 2005; Zaher and Unrau, 2007).

In general, individual proteins or ribozymes can be expressed in individual compartments by distributing genes within stable, aqueous microdroplets such that the gene:microdroplet ratio is less than 1:1. While this method is functionally similar to traditional bacterial cloning schemes (with the aqueous compartments serving as "artificial cells"), there can be around 10^{10} unique compartments per milliliter of oil phase, which is larger than is often possible for libraries expressed in *E. coli*. Because emulsion selections are carried out completely in vitro, it may also be possible to specify reaction conditions that would otherwise be unattainable in or toxic to cells.

Once a protein or ribozyme is produced from its DNA template, functional variants can be selected after further modification and/or amplification of the template, either before or after de-emulsification (Tawfik and Griffiths 1998; Ghadessy et al., 2001, 2004; Doi et al., 2004; Zheng and Roberts, 2007). For example, *Taq* polymerase variants have been selected based on the selective amplification of the *Taq* gene by translated polymerases within individual emulsion bubbles (Ghadessy et al., 2001). Alternatively, it has proven possible to selectively capture a protein phenotype and the genotype that encodes it (Sepp et al., 2002; Griffiths and Tawfik, 2003; Aharoni et al., 2005; Levy et al., 2005; Mastrobattista et al., 2005). For example, functional β-galactosidase enzymes have been selected by sorting emulsion bubbles containing larger amounts of fluorescent reaction products from emulsion bubbles containing smaller amounts of these products, and then subsequently amplifying the genes that were captured along with the fluorescence (Mastrobattista et al., 2005). Instead of sorting emulsion bubbles, it is also possible to sort beads that hold both gene and phenotype. For example, genes encoding a ribozyme ligase were immobilized on beads and emulsified. Those templates that encoded functional ribozymes were able to ligate fluorescent tags to the beads, which could be sorted from one another following de-emulsification (Levy et al., 2005). In the method described herein, a binding target is covalently attached to each DNA template. Following transcription and translation, the reaction is de-emulsified, and the translated protein is captured. Functional protein variants mediate recovery of their DNA templates by binding the target molecule through the protein-capture step. The ultimate goal of this protocol is the identification of protein sequences that bind the target molecule. For example, functional streptavidin variants that bind their biotinylated templates can be selected (Levy and Ellington, 2008; Fig. 24.6.1).

Figure 24.6.1 *(figure appears on next page)* Scheme for binding selections in in vitro compartments. (**A**) A generic template for binding selections (top), and the template for streptavidin selections (bottom) as further described in Levy and Ellington (2008). The leftmost triangle represents the target molecule attached to the template (e.g., biotin). The promoter (T7 RNA polymerase promoter) is required for transcription initiation while the ribosome binding site (RBS) enhances translation initiation. The "tag" is part of the protein sequence (a hexahistidine or His_6 tag in the current example) and enables affinity purification of the translated protein. (**B**) Selection schema showing recovery of a desired template and protein (light gray) and removal of inactive template (dark gray). From top to bottom: Compartments are formed containing no more than 1 gene. The templates are transcribed and translated to produce proteins. Some proteins will bind the target molecule conjugated to their templates. The translated proteins must retain their templates throughout the recovery and wash process. While nonbinding proteins will also be captured, they will not carry their corresponding templates with them. Captured templates will be amplified by PCR and used in subsequent rounds of selection.

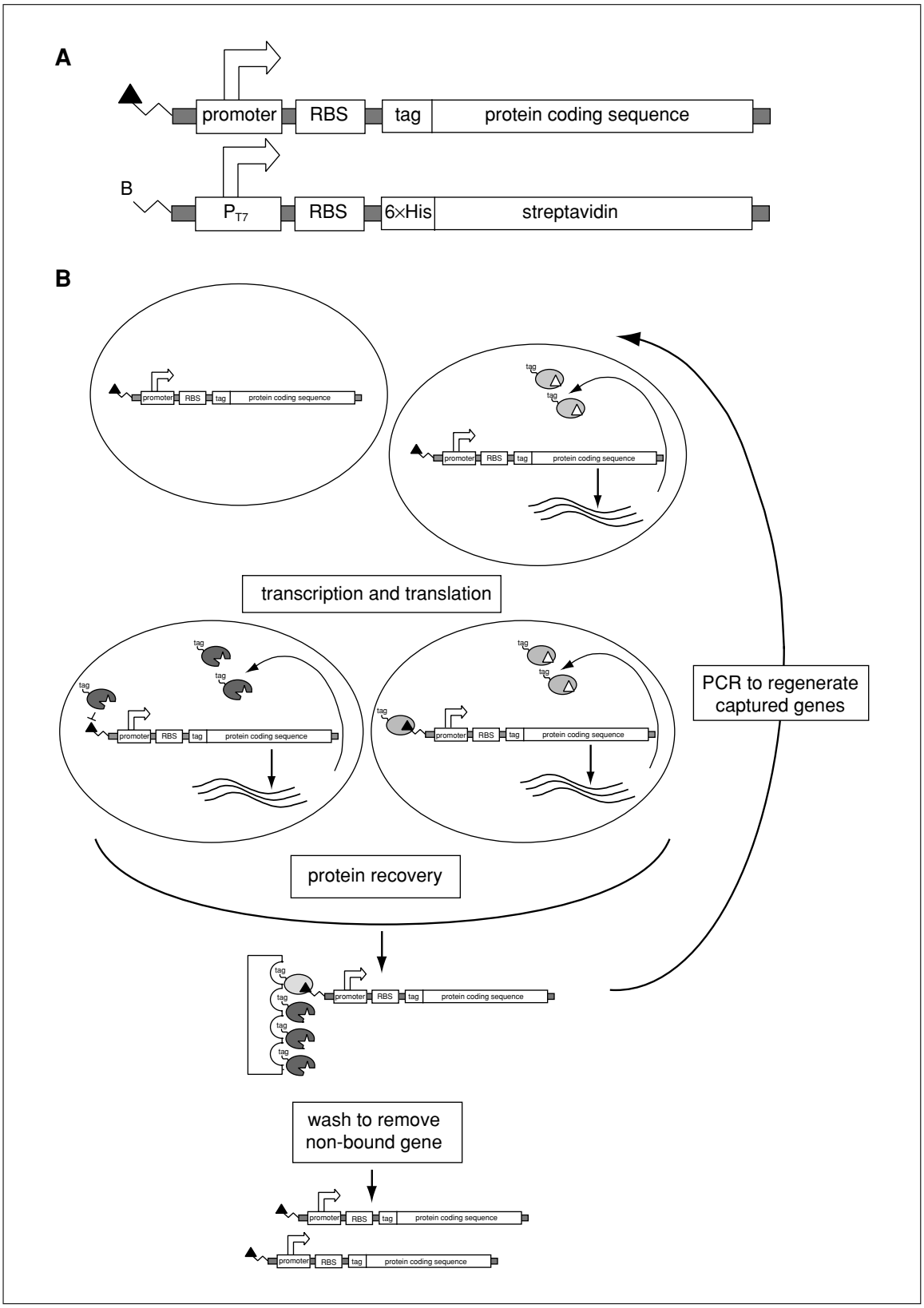

Figure 24.6.1 *(legend appears on preceding page)*

While setting up and incubating biological reactions contained within emulsions is surprisingly straightforward and only requires readily available molecular biology equipment, generating viable selection schemes takes very careful planning and troubleshooting. Not all proteins are amenable to emulsion selections, and for those that are, care must be taken to ensure that the mode and stringency of selection are appropriately matched to the capabilities of the system.

Materials

DNA of interest
Mineral oil (molecular biology grade, RNase-, DNase-, protease-free; Sigma, cat. no. M5904)
Span-80 (sorbitane monooleate; e.g., Sigma, cat. no. S6760, or Fluka, cat. no. 85548)
Tween-80
Triton X-100
Cell-free transcription and translation system, e.g., Roche RTS 100 *E. coli* HY Kit including:
E. coli lysate
Reaction mix
Amino acid mixture without methionine
Methionine
Reconstitution buffer
Tris-buffered saline (TBS; *APPENDIX 2*)
Quenching agent, e.g., 100 μM D-biotin (Sigma-Aldrich, cat. no. 47868) in TBS
Diethyl ether, H_2O-saturated
Tris-buffered saline/Tween 20 (TTBS; *APPENDIX 2*)
Anti-polyhistidine antibody bound to agarose beads (Sigma, cat. no. A5713)
Elution buffer (see recipe)

95 × 16.8 mm polypropylene (13-ml) Sarstedt tubes
1.5 and 2-ml microcentrifuge tubes
Spinplus 9.5 × 9.5 mm Teflon stir bars (VWR Scientific)
Stir plate (Corning Stirrer/Hot Plate PC-420)
90 × 50–mm (or similarly sized) glass beaker (to hold the test tube containing the emulsion)
Positive-displacement pipettors (e.g., Microman from Gilson)
30°C water bath
End-over-end rotator

Additional reagents and equipment for ethanol precipitation of DNA (*UNIT 2.1A*), the polymerase chain reaction (PCR; *UNIT 15.1*), real-time PCR (optional; *UNIT 15.8*), and agarose gel purification of DNA (*UNIT 2.6*)

Create DNA library

1. Create a gene construct that can undergo selection for binding.

   An example is given here using the gene for the streptavidin protein, which is modified to contain a T7 RNA polymerase promoter and a ribosome binding site (RBS). In addition, the amino terminus of the protein contains a hexahistidine tag that will allow subsequent recovery of the translated protein. The entire expression construct is amplified with a primer containing a biotin, so that the translated streptavidin protein can bind to its biotinylated template.

   For examples of other schemes in which proteins directly capture their own genes see Background Information.

Table 24.6.1 Cell-Free Translation Mixtures in Emulsions[a]

Lysate	Emulsion composition	References
S30 *E. coli* lysate	Mineral oil 4.5% Span-80 0.5% Tween-80	Tawfik and Griffiths (1998)
	Mineral oil 4.5% Span-80 0.5% Tween-80 0.1% Triton X-100	Levy (2008)
	Mineral oil 4.5% Span-80 0.5% Triton X-100	Griffiths (2003)
	Mineral oil 4% Abil EM90[b]	Chen (2008)
PURE[c] *E. coli* lysate	Mineral oil 4.5% Span-80 0.1% Triton X-100	Zheng (2007)
Rabbit reticulocyte lysate	Mineral oil 4% Abil EM90[b]	Ghadessy et al. (2004)
Wheat germ extract	Mineral oil 4.4% Span-85 0.6% Tween 20	Yonezawa (2003)

[a]A summary of the lysates used in in vitro compartmentalization experiments and the reagents used to emulsify them.
[b]Evonik Degussa North America (*http://www.degussa-nafta.com*).
[c]Protein synthesis Using Recombinant Elements (New England Biolabs).

2. Create a sequence library for the protein of interest.

   This is commonly done through mutagenic PCR (e.g., UNIT 8.3), but can also be done by synthesizing a gene or primer with randomized sequence positions. For example, in Fig. 24.6.1A, particular positions within a streptavidin gene were randomized via PCR with a primer containing a randomized region.

   The extent of mutagenesis should be confirmed by sequencing random clones from the unselected population. Approximately ten random clones is a good starting point. More or fewer can be sequenced at the user's discretion.

Set up the emulsion

3. For each emulsion reaction, set up a Sarstedt tube containing 1 ml of the oil-surfactant mixture described below with a Spinplus stir bar, and place on ice:

   949.5 µl mineral oil
   45 µl Span-80
   5 µl Tween-80
   0.5 µl Triton X-100.

   This oil-surfactant mixture is optimized for an E. coli S30 transcription translation lysate; for other lysates refer to Critical Parameters and Table 24.6.1. Because of the viscosity of the oil, pipetting accuracy at this step is improved by using a positive-displacement pipettor (e.g., Microman from Gilson).

4. Prepare a 50-µl in vitro transcription and translation reaction using, e.g., the Roche RTS 100 *E. coli* HY Kit:

   12 µl E. coli lysate
   10 µl reaction mix

12 µl amino acid mixture without methionine
1 µl methionine
5 µl reconstitution buffer
Template DNA
H_2O to 50 µl.

It is common to set up one experimental sample (containing the randomized library for selection) plus any relevant controls, as described in Troubleshooting, below. Keep all the reagents on ice to prevent premature initiation of transcription and translation. Proceed to step 5 as soon as possible. Other in vitro translation kits can also be used; in each instance, the amount of protein produced and the activities of proteins produced should be assayed both in solution and in emulsion (see also Troubleshooting). The amount of template DNA that should be added to the emulsion reactions differs depending on the experiment (see Critical Parameters). In general, between 10^8 and 10^{11} genes will be added to a 1-ml emulsion selection. These values correspond to 0.1 to 10 templates per aqueous compartment.

5. Move the tube containing the oil-surfactant mixture (from step 3) into a beaker containing ice water. Position the beaker so that the tube in the beaker is in the center of the magnetic stir plate and stir the mixture at 1150 rpm ("high" setting) for 1 min.

6. While stirring the oil-surfactant mixture, slowly add (drop-by-drop over 1 min) the 50-µl in vitro transcription/translation reaction from step 4 to the mixture. Continue to stir for an additional 3 min.

Accumulate protein

7. Transfer the emulsion to a 2-ml tube and incubate at 30°C for 1 to 4 hr.

The emulsified translation reaction is viscous and difficult to pipet without significant loss of material when using normal air-displacement pipettor. For this reason, we suggest using positive-displacement pipettors (e.g., Microman from Gilson) to ensure complete transfer of the reaction.

The extent of protein accumulation should be monitored by gel electrophoresis (UNIT 10.2A) and staining (UNIT 10.6) or immunoblot analysis (UNIT 10.8).

Break the emulsion

8. Add 500 µl of TBS containing 100 µM biotin (for streptavidin).

This step should stop any further transcription and translation. Addition of the (biotin) quenching agent will ensure that (streptavidin) proteins will not bind additional (biotinylated) genes after the emulsion has been broken. The increase in the volume of the aqueous phase also makes the solution easier to work with in subsequent extraction steps.

100 µM free biotin is a reasonable starting concentration. The effect of a range of concentrations (typically from micromolar to millimolar) of quencher can be tested prior to the first round of selection to more accurately determine the effect on a round of selection. As the selection progresses through multiple rounds of selection, recovery, and reamplification, with functional genes being enriched within the library, it may be desirable to increase the stringency of selection (see discussion of Selection, enrichment, and stringency under Troubleshooting). Increasing the concentration of quencher in this step is a way in which the selection stringency can be increased.

9. Add 1 ml of water-saturated diethyl ether. Vortex the reaction, then microcentrifuge 5 min at 13,000 × *g*, room temperature. Remove and discard the solvent (upper) phase. Repeat ether extraction two more times.

The ether is used to break the emulsion and remove the surfactants. Since ether is a denaturant for some proteins, the robustness of the binding protein to de-emulsification should be assayed in advance.

10. Remove any excess ether by vacuum centrifugation for 5 min at room temperature.

Recover translated proteins and bound genes

11. Add 500 µl of TTBS containing 100 µl of an anti-polyhistidine antibody agarose resin.

    The beads are listed as capable of binding 5 nmol of polyhistidine-tagged protein per 1ml of settled resin.

    The amount of resin required may have to be optimized based on how much protein is produced in the reaction and on the capacity of the resin. While the guidelines provided by suppliers are a reasonable starting point, preliminary capture experiments should always be carried out.

12. Incubate at room temperature for 30 min on an end-over-end rotator.

    End-over-end rotation may facilitate the binding of the His_6-tag to the anti-polyhistidine antibody. Stringency can be increased at this step by increasing the incubation time and thus the time during which each protein must continuously bind the target molecule attached to its template. Release and rebinding is reduced by the presence of a competitor—in this case, excess biotin.

13. Microcentrifuge the reaction 5 min at 13,000 × *g*, room temperature. Carefully remove and discard the supernatant without disturbing the agarose resin pellet.

14. Add 1 ml of TTBS to the resin. Wash the resin by gently inverting or flicking the tube. Microcentrifuge the reaction 5 min at 13,000 × *g*, room temperature. Carefully remove and discard the supernatant without disturbing the agarose resin. Repeat this wash step three times.

15. Add 400 µl of elution buffer to the agarose resin pellet. Vortex the reaction. Microcentrifuge the reaction 5 min at 13,000 × *g*, room temperature. Remove the supernatant and place it in a clean 1.5-ml microcentrifuge tube. Repeat this step once and combine the two elution fractions.

    This step denatures the anti-polyhistidine antibody and results in the release of the captured product.

16. Ethanol precipitate the recovered DNA as described in *UNIT 2.1A* and amplify the gene product by PCR as described in *UNIT 15.1*.

    In order to monitor the progress of the selection, it may be useful to carry out real-time PCR reactions relative to standards (for details on this method, see UNIT 15.8). As the selection progresses, fewer cycles should be required for amplification, the Ct value should be lower, and/or the total amount of recovered product should increase. The primers for PCR amplification should re-establish the promoter and other sequences required for expression, i.e., the entire template, as described under step 1. If additional rounds of selection are to be carried out, the binding target should again be added to the template. In the streptavidin example, this is done through PCR with a biotinylated primer.

17. Gel purify the PCR products (*UNIT 2.6*).

18. Amplify the pool of gel-purified products by PCR (*UNIT 15.1*).

19. Ethanol precipitate the amplified PCR products (*UNIT 2.1A*).

    The PCR products can be used in subsequent rounds of selection or can be cloned into vectors for sequencing and analysis. While the initial library may contain only one or a few copies of each variant, in subsequent rounds there should be multiple copies of successful variants.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Elution buffer

7 M urea
300 mM sodium acetate (add from stock of 3 M sodium acetate, pH 5.2; e.g., Sigma)
Filter through 0.2-μm filter
Store up to 6 months at room temperature

COMMENTARY

Background Information

Selections for binding proteins are only one of several different types of selections that can be carried out using the emulsion technique described in this unit. Binding proteins have been selected using two different methods. In the method described above, the binding target is covalently linked to the DNA template. Recovery of each template is dependent upon the ability of the protein it encodes to capture its gene through the target. Besides streptavidin, other binding proteins that have been selected by this method include zinc finger or p53 binding to DNA (Sepp and Choo, 2005; Fen et al., 2007). In a second approach, the emulsion is used to create template:protein linkages, which are subsequently selected for binding following de-emulsification (Doi and Yanagawa, 1999; Yonezawa et al., 2003, 2004; Bertschinger and Neri, 2004). For example, a fusion protein between a zinc finger and U1A binds to its template. Following de-emulsification, these DNA:protein chimeras were selected for their ability to bind to the U1A RNA hairpin (Chen et al., 2008). This latter method is conceptually similar to the evolution of functional proteins via mRNA display (Roberts and Szostak, 1997).

Critical Parameters

Choice of binding protein

Not all proteins are amenable to selection in emulsions, in large measure because not all proteins acquire function following translation in vitro, but also because both the emulsion and the procedures used for de-emulsification may lead to denaturation. Therefore, it is important to ensure that the wild-type protein is functional following in vitro translation, and that the protein retains function following emulsification. If a protein proves to not be particularly robust to emulsion selections, it may be possible to produce a more robust variant that is more suitable for selection by neutral drift (Bershtein et al., 2008).

Translation yield

Even when a protein can be actively translated, the yield may be too low for selection. This will be especially true for longer proteins, as cell-free translation is relatively inefficient and mRNAs can degrade in many cell lysates. While many of the critical parameters described below suggest how the amount of translation product can be improved, most successful selections will of necessity involve shorter, stable proteins. It is strongly suggested that an entire cycle of selection be carried out with the wild-type template and that yields be determined prior to undertaking a more arduous selection experiment. If only a small fraction (<10%) of the wild-type protein is recovered, then either the recovery must be optimized (see Troubleshooting) or the selection should not be attempted.

Template sequence and preparation

Protein yields are greatly affected by both the template DNA sequence and the template purification methods used. The DNA sequences required for proper translation will vary depending on the lysate type. Most lysates use a phage RNA polymerase for transcription, so the incorporation of an appropriate phage promoter is required. T7 is the most common RNA polymerase used in *E. coli*-based systems, while SP6 is more common in rabbit reticulocyte and wheat germ extracts.

Similarly, translation initiation sequences are heavily dependent on the translation system used. In *E. coli*, a Ribosome Binding Site (RBS) directs translation. In rabbit reticulocyte and wheat germ extracts, a small "Kozak" sequence can direct moderate protein expression levels. Fortunately, RBS and Kozak sequences can coexist, and thus the same template can potentially function across different translation platforms. Viral-derived

Internal Ribosome Entry Sites (IRES) can lead to higher expression levels in eukaryotic systems, but these sequences are typically ∼10 to 100 times longer than RBS and Kozak sequences (which would require them to be ordered or assembled, rather than simply designed into PCR primers), and in addition may be very specific for a particular extract. If a Kozak sequence does not provide the protein expression levels desired, the EMCV IRES has been used to increase protein production in rabbit reticulocyte lysate (Bochkov and Palmenberg, 2006) and the TMV IRES (Gallie et al., 1988; Yonezawa et al., 2003) in wheat germ lysate.

The method used for purification of PCR products can have a pronounced effect on translation yields. The authors' method of choice is phenol extraction and ethanol precipitation with sodium acetate. Other methods typically yield significantly less translation product—e.g., gel-purified PCR templates translate very poorly (∼0 to 10% the yield of phenol-extracted templates) and silica membrane spin column DNA purification yields transcription templates with only modest translation efficiency (∼25% that of phenol-extracted templates; unpub. observ.).

While plasmids may prove to be better templates for translation, the capture method must be appropriately modified. For example, a zinc finger protein could capture a plasmid containing a corresponding binding site.

Capture method

Following de-emulsification, the protein product must be captured. In the example protocol described in this unit, capture is mediated by a hexahistidine tag on the protein. A FLAG tag is another common sequence that can be substituted for the hexahistidine tag. Both systems rely on commercially available affinity purification reagents. During selections not involving the directed evolution of streptavidin, the streptavidin:biotin couple can potentially be used to affinity purify proteins following de-emulsification, in place of the His tag. For each of these systems, the key will be to ensure that the capture and retention of the protein product is efficient. The procedure should be performed with the wild-type protein prior to setting up a selection experiment.

Cell-free translation

There are a variety of translation lysates available that are compatible with protein production in emulsions. The most commonly used lysates are extracts from *E. coli* (so-called S30 extracts), from rabbit reticulocytes, and from wheat germ. The PURE system (Protein synthesis Using Recombinant Elements) contains individually purified transcription and translation components from *E. coli* (Shimizu et al., 2005), and has also been used to synthesize proteins in emulsions (Zheng, 2007). However, it is critically important that each lysate be used with an emulsion protocol that is appropriate for it (Table 24.6.1). The protocol described in this unit is very specific for *E. coli*. Other literature should be consulted for emulsion selections in rabbit reticulocyte lysate (Ghadessy and Hollinger, 2004) or in wheat germ (Yonezawa et al., 2003). Regardless of type, commercial or freshly prepared lysates should be aliquotted and stored at −80°C. Freeze/thaw cycles quickly cause inactivation of the lysate and should be avoided for best results.

Emulsion technique

The procedure used to form the emulsion will greatly affect the lysate activity. The viscosity of the reagents used to create emulsions makes it difficult to pipet accurately with standard air-displacement pipettors, and thus the use of positive displacement pipettors (such as the Microman series from Gilson) is strongly suggested. Care should be taken to set up the lysate reaction on ice and to immediately emulsify the reaction. This will prevent transcription and translation in solution prior to emulsification. This is important because, if functional genes are produced outside of compartments, they can potentially capture templates that are not their own.

The number of genes per compartment is a variable that will affect the course of the selection. Multiple genes per compartment will allow a great population of variants at the outset, but will slow the overall progress of the selection. An average of one gene or less per compartment should allow for greater enrichment per round. Therefore, a strategy in which the number of genes per compartment is progressively decreased may allow the largest number of variants to be efficiently plumbed. If the stringency of selection is increased as the number of genes per compartment is decreased, the overall enrichment may be synergistically enhanced.

Troubleshooting

The success of emulsion protein selections can be affected by a variety of problems, but assuming that the experiment has been

designed properly (see Critical Parameters), these are almost always related to problems with either translation or emulsion. For example, it is not possible to select for activity if there is a problem with protein expression or if the expressed protein is inactive. Therefore, it is critical to not only verify expression and activity prior to initiating a selection experiment, but also during the course of the selection in order to ensure that "futile cycles" are not being performed. Selections can also fail when there is inefficient or nonspecific recovery of genes.

Real-time PCR assays

The only signal that will generally be apparent following a round of selection is the PCR product that arises. In order to ensure that this PCR product is indeed due to the selection, it is always recommended to carry out control reactions. A negative control might be a template encoding a nonfunctional (truncated) protein, while a positive control might be a template encoding the wild-type protein.

Protein production

If little or no DNA is recovered from an emulsion (as determined by real-time PCR), it is possible that little protein has been produced. Unfortunately, there is usually too little protein produced in the context of an emulsion selection to assay directly. If a given round of selection has failed, then the quality of the template should be assessed by carrying out in vitro transcription and translation in solution (i.e., in the absence of emulsification) and confirming the presence of a protein product by gel electrophoresis and staining or immunoblot analysis. If very small amounts of protein are produced or if there is no tag that can be used for immunoblot analysis, proteins can be labeled with radioactive amino acids (such as [^{35}S]methionine) during translation.

If adequate protein is produced in solution, but not in the emulsion, it is possible that the emulsion reaction itself is poisoning protein production or that protein is lost during de-emulsification. It is possible to determine whether this is the case by performing three translation reactions in parallel. The first reaction is a standard translation in solution. The second is the same as the first, but emulsified *following* translation. The third reaction is translation in emulsion. Following ether extraction of reactions 2 and 3, the amount of protein produced in each reaction can be compared by immunoblotting or by comparing the amount of radiolabeled protein produced.

In order to increase protein production, it may be necessary to try different lysate sources and different emulsification techniques (see Table 24.6.1). If little or no protein is carried through the aqueous phase following ether extraction, alternative methods for breaking the emulsion can be used. Chloroform, hexanes, and other organic solvents can be used to break emulsions, and their efficiency can be compared to that of ether. The protein activity remaining after breaking the emulsion decreases with every additional ether extraction, and so it may be beneficial to use fewer ether extractions. However, residual amounts of emulsifying agents can interfere with subsequent amplification.

Even if a protein is translated, it is often unclear whether the protein is active, especially in emulsion relative to aqueous solution. Protein activity in emulsion can be difficult to assess. Often, the most effective assay involves just testing DNA recovery and amplification in the selection scheme itself, especially since real-time PCR is often much more sensitive than many enzyme assays.

Emulsion formation

While emulsions are extremely easy to prepare, they can also be heterogeneous and idiosyncratic. Individual published methods have variations in the emulsion composition and the mechanics of emulsion formation. Inspecting the emulsion under a microscope can help establish the size and size distribution of the aqueous compartments generated. Unfortunately, it is unclear what the optimum size of an aqueous bubble is for the production and activity of a given protein. A general measure for determining the activity of the transcription and translation reaction within an aqueous compartment is to translate a fluorescent protein such as eGFP and verify protein production by fluorescence microscopy or a fluorimeter.

Selection, enrichment, and stringency

The identification of functional variants from a large and diverse starting library by directed evolution can be a powerful method, but specific implementations inherently differ due to differences in function selected for. Because of this, testing the selection procedure prior to attempting selection with a randomized library is important both to ensure that the designed scheme works as intended and to determine the likely enrichment per cycle of selection, and to evaluate the stringency of selection. To verify that protein activity in emulsion leads to an

enrichment of active variants in a population, it is common to perform a "mock selection" using a population of only two variants: one variant with normal activity and one (truncated or mutated) variant with low or nonexistent activity. The active variant is mixed with the inactive variant at various ratios (e.g., 1:10, 1:100, 1:1,000), and a single cycle of selection is carried out. The level of enrichment relative to standards is determined by real-time PCR or by cloning and sequencing. If substantial (>10-fold) enrichment is not readily apparent and active protein is being produced in emulsion (see above), individual steps of the recovery and reamplification procedure should be investigated for either false negative signals (through unintended loss of active variants) or false positive signals (through the nonspecific retention or amplification of inactive variants). For example, to test whether the recovery of genes following de-emulsification is efficient, a wild-type protein can be added prior to emulsification and the fraction of genes recovered can be quantified by real-time PCR. A lack of enrichment could also be due to initially overloading aqueous compartments with multiple variants, most of which may be inactive. Therefore, it is prudent to verify whether that there are only a small number of genes (0 to 2) per compartment.

The stringency of a selection refers to the level of function required for an individual member of the library to be propagated through the selection cycle for characterization and/or further cycles or rounds of selection. If a round of selection cannot separate functional from nonfunctional variants, it is not sufficiently stringent, and there cannot be enrichment. If a functional variant cannot be propagated through a round of selection, the selection is too stringent. The appropriate level of stringency and the mechanisms by which stringency can be modulated will depend on the specific selection. As a selection progresses and the diverse starting library becomes less diverse and more functional, it may be necessary to increase the selection stringency to allow the variants with the highest function to be further enriched from those with moderate function. In the context of streptavidin binding to biotin, selection stringency was set by the time required after de-emulsification for protein recovery prior to amplification (Basic Protocol, steps 11 through 14). Stringency is increased by increasing the incubation time during which binding must be maintained and through the presence of binding competitors.

Anticipated Results

For the selection described in this unit, where only a few amino acids were randomized (Levy and Ellington, 2008), the entire selection was completed within only a few (two to seven) rounds. For rarer phenotypes or less robust selections, more cycles may be required. In general, though, it is anticipated that the real-time PCR signal (Ct) will decrease over the course of several rounds. If no decrease is seen or if the signal is highly variable, then some action is likely required (see Troubleshooting). As has previously been seen for phage-display selections, another indication of the success of the selection is winnowing of the pool, which can be determined by sequencing. However, in the absence of a decrease in real-time PCR signal, such a narrowing of the pool is suspect.

Time Considerations

Because each new emulsion selection generally requires the development of new protocols, the time required for initial optimization and troubleshooting is generally quite large (on the order of months). However, once the selection scheme has been optimized, each round of selection can be performed in less than 2 days. The number of rounds of selection necessary will depend on the relative abundance of the desired protein phenotype, the size of the pool, the enrichment per round, and the stringency of selection.

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